SUMMARY

1. The kinetics of the activation of rat-liver arylamine sulphokinase by 17-oxo steroids has been investigated, and it has been shown that they are consistent with the mechanism of the activation's being of a mixed type in which partial competitive and partial non-competitive components are combined.

2. The specificity of the activating effect has been studied and it has been shown that the structural requirements for activation are fairly stringent. A double bond in the D ring of the 17-oxo steroid gives an inactive compound. A substituent in the 16 position (α or β) may or may not eliminate the effect of the 17-oxo group.

3. 16-Substituted 17-oxo steroids which are without effect or which inhibit arylamine sulphokinase may still combine with the enzyme but because of the relative magnitudes of the various velocity and dissociation constants activation does not occur.

4. The structures of the A and C rings of the steroid influence the activating effects of 17-oxo steroids.

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Calcium Binding by Rat Submaxillary Glands *in vitro*

By R. H. DREISBACH

Department of Pharmacology, Stanford University School of Medicine, Palo Alto, Calif., U.S.A.

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Rat submaxillary glands contain about four times as much calcium as other soft tissues such as liver and kidney (Dreisbach, 1957), and approximately 50% of this calcium is lost after 2 hr. of maximal pilocarpine stimulation of secretion (Dreisbach, 1959a). One possibility is that this labile calcium in rat submaxillary gland is due to the presence of calcium-binding components in the gland which are different, either qualitatively or quantitatively, from the calcium-binding components in plasma. If sufficient binding material is present in the gland, the intracellular unbound calcium concentration could be the same as that present in the extracellular fluid, and no concentration gradient across the cell membrane would occur. The high tissue calcium could thus be maintained without a specific concentrating mechanism.

The present report describes the effects of variations in pH, temperature and calcium concentration on calcium binding *in vitro* by rat submaxillary glands either as slices or as homogenates.

MATERIALS AND METHODS

Submaxillary glands were removed under chloroform anaesthesia from adult Long-Evans rats of either sex. Except for experiments in which glands were used immediately, glands were stored in screw-capped vials at -20° for at least 1 week before use.

Preparation of homogenates. Glands were disrupted in batches of 0.5 g. by grinding for 3 min. in a glass-walled homogenizer of 10 ml. capacity with Teflon pestle. The pestle was driven at 1000 rev./min. and the apparatus was cooled in ice. The medium for disruption consisted of 0.25 M-sucrose or 0.15 M-NaCl, by using 9 vol. of medium for

each vol. of submaxillary gland. The resulting suspension was filtered through gauze and then dialysed for 16 hr. at 2° against a large volume of 0.15 M-NaCl solution in order to remove dialysable calcium-binding substances. Homogenate suspensions after dialysis were adjusted to a final concentration of 0.01 M-sodium barbital for buffering at pH 7.4 and 8.5 or of 0.01 M-sodium acetate for buffering at pH 4.0 and 5.5. The pH, as indicated by a Beckman Zeromatic pH meter, was adjusted with 0.15 M-HCl or 0.15 M-NaOH. Ionic strength of all solutions was maintained at 0.15. Barbital and acetate buffers were used in view of the negligible capacity to bind calcium shown by these substances (Shubert, 1952).

Equilibrium dialysis. This was done in equipment similar to that used by Teresi & Luck (1952), in which the maximum distance from the dialysis membrane in each chamber did not exceed 3 mm. and the volume on each side of the membrane was approximately 1.0 ml. Equilibration was carried out for 16 hr. at 2° with slow shaking. When CaCl_2 solutions were dialysed against NaCl solutions in these cells, equilibrium was reached in 4 hr. and, in preliminary trials with tissue suspensions, the equilibrium did not change after 6 hr. The pH did not change during the experiment.

Ultrafiltration. The time required for equilibrium dialysis allowed decomposition to occur in the tissue suspensions when equilibration was done at 30°. For this reason, binding by homogenates at elevated temperatures was determined by ultrafiltration by using the method of Toribara, Terepka & Dewey (1957). The use of a 5% CO_2 atmosphere was unnecessary since the pH of buffered homogenates did not change in the presence of air.

Tissue slices. Tissue slices were prepared from fresh glands or thawed frozen glands, with the tissue slicer described by Stadie & Riggs (1944). Slices were approximately 0.4 mm. thick and they were shaken slowly in air for 2 hr. for equilibration. Preliminary experiments indicated that the amount of calcium in slices did not change after 2 hr. The medium for tissue slices had the following composition: KCl, 4.0 mM; sodium barbital buffer (pH 7–8), 10.0 mM, or sodium acetate buffer (pH 4–6), 10.0 mM; MgCl_2 , 0.1 mM; dextrose, 5.0 mM; sufficient CaCl_2 to achieve the indicated concentration of calcium; NaCl to give I 0.15. The ratio of tissue to medium was approximately 1:25 in order to minimize the effects on calcium distribution of binding substances escaping from the slices. After incubation, tissue slices were blotted to remove excess of media before weighing. Dry weight was determined after 16 hr. at 105°.

Chemical methods. Total nitrogen analyses were done by the method of Lang (1958). Calcium was determined by flame photometry (Dreisbach, 1959b) or by EDTA titration (Grette, 1953); differences between duplicate analyses did not exceed 5% for either method. Nitrogen analysis of tissue homogenates was done before distribution for equilibration dialysis or ultrafiltration. After equilibrium dialysis, samples from both sides of the membrane were analysed for calcium after precipitation with 10% trichloroacetic acid. Calcium concentrations of all media after equilibration were determined by comparison with the calcium standard used for the analysis of tissue slices and homogenates. The chloride concentration on both sides of the membrane, after equilibrium dialysis, was determined by AgNO_3 titration with the amperometric indicator

system of Cotlove, Trantham & Bowman (1958). The distribution ratio, $\lambda = (\text{Cl}^-)_{\text{outside}}/(\text{Cl}^-)_{\text{inside}}$, was never more than 1.01 at I 0.15. Since the amount of chloride bound by submaxillary-gland homogenates was unknown, the Donnan effect was ignored in calculations of bound calcium.

Calculations. Since the intracellular or intraparticle unbound calcium could not be determined directly, certain assumptions were necessary in order to calculate bound calcium. The amount of calcium bound in slices was considered to be the amount present in the tissue in excess of the amount which would be present if tissue water contained the same concentration as the medium. In studies on homogenates, the dialysate or ultrafiltrate was assumed to represent the aqueous phase of the homogenate. Some of the excess of calcium present in the slices or homogenates could also have been maintained by processes other than binding, but this possibility has not been studied in the present experiments.

RESULTS

Relation of calcium binding by rat-submaxillary-gland slices or homogenates in vitro to calcium concentration. When submaxillary glands were disrupted in calcium-free media and then dialysed overnight against a large volume of calcium-free 0.15 M-NaCl solution, the final calcium concentration of the homogenate was always less than 0.01 mM. Thus no appreciable quantity of non-dissociable calcium was present in the homogenate preparation.

When submaxillary-gland homogenates were dialysed at 2° against NaCl solutions containing progressively greater amounts of Ca^{2+} ions at pH 7.4 or 8.5, the apparent amount of calcium bound in the homogenate increased progressively as shown by the top curve in Fig. 1. Ultrafiltration at 12° of homogenates containing increasing amounts of calcium also showed an increase in the amount of calcium bound as the concentration of calcium in the filtrate went up (Fig. 2).

In order to ascertain whether the extent of binding was the same in homogenates as in less disrupted tissues, the calcium present in tissue slices was also determined after exposure to various concentrations of calcium in more physiological media (Table 1). The results in these various experiments indicate that at 2–12° the apparent amount of calcium bound/mg. of N in relation to unbound calcium is nearly the same whether determined in homogenates in NaCl– CaCl_2 solutions by equilibrium dialysis or ultrafiltration, or in slices suspended in more physiological media. The homogenization procedure thus did not affect appreciably the relationship of calcium-binding capacity to nitrogen content. However, dialysable nitrogenous substances that bound calcium in the same ratio could have been lost during preparation of the tissue homogenates without changing the apparent amount of calcium bound.

Effect of temperature on calcium binding by sub-maxillary-gland slices and homogenates. The μ moles of calcium bound/mg. of N for slices of fresh gland equilibrated at 30° and at 2° are given in Table 1. The slices bound essentially the same amount of calcium/mg. of N at either temperature. However, a small effect of temperature would have been obscured by the relatively large error in the procedure. The frozen gland slices equilibrated at 30° appeared to bind somewhat more calcium than the fresh gland slices equilibrated at the same temperature when the amount of calcium bound was related to nitrogen content. On the other hand, when the amount of calcium bound was recalculated on the basis of dry weight (Table 1), in no instance did the frozen gland slices bind more calcium than did the fresh gland slices.

The amount of calcium bound by submaxillary-gland homogenates at 12° and 30° as determined by ultrafiltration is shown in Fig. 2. Duplicate samples at each concentration fell within the diameter of the circle shown. The data indicate a slight increase in binding at 12° as compared with 30°.

Effect of pH on calcium binding by rat sub-maxillary gland. Fig. 1 illustrates the effect of altering pH on the binding of calcium in rat-submaxillary-gland homogenates as determined by equilibrium dialysis at 2° with various concentrations of calcium. The amounts of calcium bound at pH 7.4 or 8.5 were not significantly different,

Fig. 1. Effect of calcium concentration and pH on calcium binding by rat-submaxillary-gland homogenates as determined by equilibrium dialysis at 2°: □, pH 8.5; ○, pH 7.4; ●, pH 5.5; ■, pH 4.0; I 0.15. Each point represents one determination.

Fig. 2. Effect of temperature and calcium concentration on non-ultrafiltrable calcium in rat-submaxillary-gland homogenates. Barbiturate buffer, pH 7.4, I 0.15; temperature: ●, 12°; ○, 30°.

Table 1. *Effect of temperature of incubation and prior freezing on binding of calcium by rat-submaxillary-gland slices*

Values given are means $\pm$ s.e. for five determinations at each concentration. I, 0.15; buffer, 0.01 M-barbital, pH 7.4; equilibration time 2 hr. Frozen gland refers to gland that had been frozen and thawed before slicing.

Concn. of Ca ²⁺ (mM)	Ca bound (μ moles/mg. of N)			Ca bound (μ moles/g. dry wt.)		
	Fresh gland 30°	Fresh gland 2°	Frozen gland 30°	Fresh gland 30°	Fresh gland 2°	Frozen gland 30°
1.4	0.61 $\pm$ 0.08	0.45 $\pm$ 0.03	0.82 $\pm$ 0.07	24.2 $\pm$ 2.8	20.8 $\pm$ 2.9	18.3 $\pm$ 1.4
2.4	0.74 $\pm$ 0.07	0.74 $\pm$ 0.08	1.27 $\pm$ 0.14	29.1 $\pm$ 2.7	34.3 $\pm$ 0.5	22.5 $\pm$ 3.9
5.0	1.44 $\pm$ 0.22	1.11 $\pm$ 0.03	2.26 $\pm$ 0.33	45.4 $\pm$ 6.2	54.3 $\pm$ 2.8	44.7 $\pm$ 11.1
10.0	2.09 $\pm$ 0.38	2.18 $\pm$ 0.22	2.80 $\pm$ 0.04	63.1 $\pm$ 9.9	104.9 $\pm$ 6.8	54.9 $\pm$ 7.2

Fig. 3. Effect of pH on calcium binding by rat-submaxillary-gland slices at 2°. Each point represents one determination; $\log [\text{Ca}^{2+}]$ free, -2.40 ; glands were previously frozen; I 0.15.

whereas binding at pH 5.5 was distinctly less but still appreciable. Binding of calcium at pH 4.0 appeared to be only slight.

The effect of pH on calcium binding by rat-submaxillary-gland slices is shown in Fig. 3. Glands were frozen at -20° and then thawed before slicing. Equilibration was carried out for 3 hr. at 2°. The amount of calcium bound/mg. of N appears to increase in linear fashion with change in pH from 4 to 6 but does not increase appreciably above pH 7. The amount of calcium bound by slices at different pH values was similar to that found for homogenates. Thus the method of tissue preparation did not alter appreciably the relation of binding to pH.

DISCUSSION

The present experiments reveal both qualitative and quantitative differences in some aspects of calcium binding by rat-submaxillary-gland homogenates and slices as compared with human serum proteins (McLean & Hastings, 1935), human serum albumin (Martin & Perkins, 1950) or bovine serum albumin (Katz & Klotz, 1953) when measurements are made at I 0.15. Complete reversibility of binding apparently occurs with all these preparations but rat submaxillary gland binds more calcium than do serum proteins. For example, the data given by McLean & Hastings (1935) for patients with a serum calcium ion concentration of 1.30 m-

moles/kg. of water indicates calcium binding of 0.13–0.18 $\mu\text{mole/mg. of nitrogen}$. Martin & Perkins (1950) measured at pH 6.3–6.8 the calcium binding of human serum albumin prepared by ethanol or methanol fractionation. They obtained 0.025–0.118 $\mu\text{mole of calcium bound/mg. of nitrogen}$ at a calcium ion concentration of 3.5–4.7 mm with most of the values near the upper figure. In contrast, submaxillary-gland preparations bound 0.41–0.82 $\mu\text{mole of calcium/mg. of nitrogen}$ at a calcium concentration of 1.3–1.4 mm and 1.0–2.3 μmoles at 5.0 mm. Thus the calcium binding *in vitro* by these rat-submaxillary-gland preparations is several-fold higher than has been reported for human serum proteins and is probably sufficient to account for the 7–10 m-moles of calcium/kg. wet wt. found in the gland (Dreisbach, 1959a). Whether the salivary glands are unique in this respect among the soft tissues of the rat is unknown, since calcium binding by other tissue preparations under similar conditions has apparently not been studied.

The substance or substances responsible for calcium binding in rat submaxillary glands may interact with calcium according to the Law of Mass Action in a manner similar to that described for the binding of calcium by human serum proteins by McLean & Hastings (1935) or for bovine serum albumin by Katz & Klotz (1953), since the amount of calcium bound increases with increasing Ca^{2+} ion concentration. However, no such formulation was attempted in view of the inhomogeneity of the gland preparations.

Lowering the temperature at which binding was measured from 30° to 12° appeared to increase slightly the amount of calcium bound as determined by ultrafiltration. However, gland slices showed no difference in binding at 30° or 2° . Thus the properties of the binding substances in rat submaxillary gland are different from those present in human serum, which shows a decrease in binding at lower temperatures (Toribara *et al.* 1957).

The effect of pH on calcium binding *in vitro* by submaxillary gland appears to be similar to that occurring in frog-muscle brei (Weimer, 1953) and to be different from bovine serum albumin (Katz & Klotz, 1953). In the experiments of Katz & Klotz (1953), there was somewhat less binding by bovine serum albumin at pH 7.6 than at 8.8 and almost no binding at pH 5.5. Frog-muscle brei (Weimer, 1953) showed maximum binding of calcium (97%) at pH 7.8–5.4, falling to 50% at pH 5.0 and to 10% at pH 4.7. Submaxillary-gland preparations showed little change in binding at pH 8.5–7.4, falling to 50% of the initial amount at pH 5.0, and to approximately 20% at pH 4.0. Thus the pH at which binding ability *in vitro* was reduced to half of the initial amount was considerably lower for

both frog-muscle brei and submaxillary gland than for bovine serum albumin.

Little is known about the nature of the binding material present in the rat submaxillary gland. In a comparison of binding by slices of fresh or previously frozen glands, the calcium bound/mg. of nitrogen was higher in previously frozen glands than in fresh glands, whereas calcium bound/g. dry wt. was not different in the two preparations. This discrepancy may arise as a result of a greater loss of non-calcium-binding nitrogen-containing compounds from the frozen and thawed glands than from the fresh glands. The methods used to study calcium binding in these experiments measure only the fraction of calcium bound to molecules that do not dialyse or that cannot escape from slices, whereas calcium may also be bound to dialysable components. For example, human saliva apparently contains dialysable substances that account for most of the calcium binding (Dreisbach, 1960). Unfortunately, no method available at the present time will allow direct determination of the relative amount of Ca^{2+} ions in systems as complex as the present tissue preparations.

Another possible way by which at least a part of the excess of calcium could be maintained in the submaxillary gland is by a metabolism-dependent transport mechanism such as the one that maintains intracellular potassium. However, the close correlation between the amount of calcium apparently bound by the submaxillary-gland preparations used in the present experiments, whether fresh or previously frozen and at temperatures from 2° to 30° , indicates that such a mechanism probably does not play a part in the maintenance of the concentrations of calcium found in these studies *in vitro*. This deduction agrees with the data of Schachter, Dowdle & Schenker (1960). These workers have shown that rat-submaxillary-gland slices do not actively accumulate ^{45}Ca *in vitro* in contrast with small intestine, some parts of which can maintain a cell concentration of ^{45}Ca seven times as great as that present in the media.

SUMMARY

1. The binding of calcium by rat-submaxillary-gland homogenates *in vitro* was completely reversible in the presence of sodium chloride at an ionic strength of 0.15. The increase in calcium bound by slices or homogenates was approximately log-linear over the range 1–10 mM- Ca^{2+} .

2. Slices or homogenates of rat submaxillary glands bind approximately three times as much calcium as do serum proteins at the same ionic strength.

3. The range of temperature from 2° to 30° had little effect on calcium binding by rat-submaxillary-gland slices or homogenates.

4. Change in pH from 7.4 to 5.0 decreased the amount of calcium bound by rat-submaxillary-gland homogenates to approximately one-half the initial amount, whereas increase of pH from 7.4 to 8.5 did not increase significantly the amount of calcium bound.

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