CAPACITATION INDUCING ACTIVITY OF SERUM ALBUMIN IN FERTILIZATION OF MOUSE OVA *IN VITRO*

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The role of serum albumin in *in vitro* fertilization of mouse ova was studied relating to the fertilization inhibitory activity of Zn^{2+} . The time required for mouse sperm capacitation was dependent on the concentration of serum albumin which was added to the medium. The majority of Zn^{2+} added to the medium was shown to be bound to serum albumin (95% out of $200\ \mu\text{M}$) by means of equilibrium dialysis. Furthermore, it was suggested that the serum albumin was necessary for the initiation of the sperm capacitation process by the periodic observation of sperm penetration through zona pellucida.

Keywords — *in vitro* fertilization; mouse ova; capacitation; zinc ion; serum albumin; mouse sperm

In mammals, ejaculated spermatozoa require a physiological change called capacitation^{1,2)} *in vivo* or *in vitro* followed by a morphological change known as acrosome reaction before they acquire a fertilizing ability.³⁾ Protein, involved in the *in vitro* induction of the hamster sperm acrosome reaction, was purified from bovine follicular fluid^{4,5)} or bovine serum,⁶⁾ and researchers have claimed that the serum albumin plays a significant role. It was also reported that the commercially available albumin was also effective in the induction of an acrosome reaction.⁶⁾ However, the role of albumin throughout the fertilization process was not clearly identified. In the present paper, we examined whether albumin is required in the induction of an acrosome reaction or required in the induction of sperm capacitation by means of periodic observation of fertilization of mouse ova *in vitro*.

To investigate the mechanism of the sperm capacitation phenomenon, the capacitation inhibitory activity of Zn^{2+} reported by Aonuma^{7,8)} was also studied in relation to capacitation inducing activity of serum albumin.

MATERIALS AND METHODS

Preparation of Sperm Suspension and Ova —

The standard medium used for the experiments was a modified Krebs Ringer bicarbonate solution (m-KRB) which was reported by Toyoda *et al.*⁹⁾ The amount of NaHCO_3 was increased from 2.01 to 5.00 g/l to bring the pH level from 7.4 to 8.0, a value shown as more suitable for *in vitro* fertilization of mouse ova in our laboratory.¹⁰⁾ The spermatozoa were obtained from the cauda epididymidis of mature ddY mice (weighing 35–40 g) as previously reported.¹¹⁾ The ova were obtained from 4–5 week old ddY mice injected intraperitoneally with 5 i.u. PMSG (Teikoku Zoki) 48 h before administration of 5 i.u. hCG (Teikoku Zoki). The mice were killed 14–16 h after hCG injection and the ova in cumulus cells were recovered by puncturing the ampullar portion of the oviduct with a needle. The collected ova were washed twice with 10 ml medium and used for the experiments.

Washing of the sperm suspension was done by centrifugation. Ficoll (Pharmacia) was dissolved in an albumin-free medium (7.5% w/v). The sperm suspension (0.8 ml) was layered carefully on top of a Ficoll containing medium (5 ml) in a measuring test tube and centrifuged at $500 \times g$ for 10 min at room temperature. After the centrifugation, the upper part of the solution

was sucked out with an aspirator, leaving 0.2 ml of aliquot. After checking the sperm concentration, (the sperm suspension was diluted if necessary) the washed sperm were added to ova.

Effects of Albumin and Various Proteins on in Vitro Fertilization of Mouse Ova—The proteins used in the experiments were bovine serum albumin (Sigma: Fr.V), mouse serum albumin (Sigma: Fr.V), ovalbumin (Sigma: chicken, Grade V), bovine γ -globulin (Sigma: Fr.II) and casein from bovine milk (Merck: essentially "vitamin-free"). To examine the effect of various concentrations of albumin, a 10% albumin solution in KRB was prepared and appropriate amounts of aliquots were added to the albumin-free m-KRB.

Periodic observation of the fertilization was made by preparing several test tubes containing ova which were identically inseminated. After a certain period of time, one of these tubes was subjected to the observation for the assessment of fertilization immediately after the hyaluronidase treatment.

Zn²⁺ Binding to Albumin—Zn²⁺ was measured by a Hitachi Perkin-Elmer atomic absorption spectrophotometer (Type 303). Equilibrium dialysis was done by placing 5 ml of albumin solution (4 mg/ml) in seamless cellulose tubing

(Visking Company) and dialyzing against 1000 ml of 50 mM Tris-HCl buffer pH 8.0 containing 12.5, 25, 50, 100 and 200 μ M of Zn²⁺ for 96 h. At the end of the dialysis, the Zn²⁺ concentrations both inside and outside of the tubing were measured.

Zn²⁺ Binding to Spermatozoa—⁶⁵ZnCl₂ (specific radioactivity, 3.65 mCi/mg Zn, radiochemical purity, 99%) was purchased from Japan Isotope Association. Epididymal and capacitated spermatozoa, prepared in 1 ml of m-KRB solution ($0.8-1.6 \times 10^6$ sperm/ml), were mixed with equal volume of radio active Zn²⁺ containing m-KRB and incubated for 10 min at 37°C (final 250 μ M Zn²⁺, 5 μ Ci/ml). After the incubation, sperms were washed by centrifugation as described before. However, in this experiment, centrifugation was done at 500 g $\times$ 5 min followed by 1200 g $\times$ 10 min and the supernatant was sucked out to leave 1.0 ml of aliquot. The remaining aliquot (1 ml) was transferred to another tube before counting the radio activity by Aloka Auto Gamma System (Type ARC-251). The control experiments ($n=6$) were done in the same manner without spermatozoa and the radio activity found in the 1 ml of the aliquot which was at the bottom of the tube (235 ± 34 cpm) was subtracted from that of the

TABLE I. *Effect of Various Concentrations of Bovine Serum Albumin on Fertilization of Mouse Ova in Vitro*

Concentration of BSA in fertilization medium	No. of experiments	Total no. of ova examined	Ova penetrated ^{a)} (%)
0 mg/ml	3	47	0
0.25	3	54	2 $\pm$ 2
0.50	5	97	28 $\pm$ 14
1.00	5	85	56 $\pm$ 13
2.00	5	85	60 $\pm$ 9
4.00	5	83	74 $\pm$ 6
8.00	3	46	71 $\pm$ 11

a) Each value represents the mean $\pm$ S.E. of 3 to 5 independent tests. To avoid the preliminary contact with albumin, spermatozoa used in these experiments were prepared in albumin-free m-KRB. Assessments for fertilization were done by observing the sperm penetration of zona pellucida or sperm fusion with vitellus 90 min after the sperm insemination.

sperm containing experiments.

RESULTS

The effect of albumin concentration in the

medium on the fertilization of mouse ova *in vitro* was studied. The most commonly used medium in *in vitro* fertilization systems usually contains 4 mg/ml albumin. When the concentration was re-

TABLE II. *Effects of Various Proteins on Fertilization of Mouse Ova in Vitro*

Sample	No. of experiments	Total no. of ova examined	Ova penetrated ^{a)} (%)
Bovine serum albumin	5	92	68 ± 9
Mouse serum albumin	5	92	71 ± 7
Chicken egg albumin	5	82	0
Bovine γ -globulin	5	87	0
Casein from bovine milk	5	80	0

a) Each value represents the mean $\pm$ S.E. of 5 independent tests. Ova were deposited in the medium containing various proteins (4 mg/ml). Spermatozoa prepared in protein-free m-KRB were added to the ova and observed for the assessment of fertilization 90 min after the insemination.

TABLE III. *Relationship of Albumin Concentration and Sperm Penetration Time*

Concentration of bovine serum albumin in culture medium	Fertilization (%) ^{a)}			
	Time after insemination (h)			
	0.5	1.0	1.5	5.0
4.0 mg/ml	0	55 ± 13	62 ± 8	65 ± 10
0.4 mg/ml	0	0	35 ± 1	66 ± 7

a) Each value represents mean $\pm$ S.E. of 4 independent tests. Total no. of ova examined were 69 to 88 in each group. In the observation done at 5 h after insemination, the formation of pronuclei was also checked as well as the sperm penetration.

TABLE IV. *Recovery of Fertilization Rate in Zn²⁺ Containing Medium by Supplement of Albumin*

Concentration of bovine serum albumin in culture medium	Concentration of Zn ²⁺	No. of experiments	Total no. of ova examined	Ova penetrated ^{a)} (%)
4 mg/ml	0 μ M	6	90	71 ± 7
4	200	6	95	0
8	200	6	99	0
16	200	5	88	41 ± 13
32	200	5	84	68 ± 10

a) Each value represents mean $\pm$ S.E. ZnCl₂ was dissolved in distilled water at a concentration of 5 mM and an aliquot was added to each medium shortly before the sperm insemination. Sperm suspension was made in m-KRB and the assessment of fertilization was done 90 min after the insemination.

duced to 0.5 mg/ml, the fertilization rate decreased significantly. We found no significant effect on the fertilization rate by raising the concentration of albumin above 4 mg/ml (Table I). As shown in Table I, albumin was essential for the fertilization of mouse ova *in vitro*. Therefore, the specificity of the albumin effect was investigated. Mouse albumin was also found to be effective. However, globulin, casein and ovalbumin were found to be completely ineffective.

FIG. 1. Scatchard Plots for the Interaction of Zn^{2+} with Bovine Serum Albumin at Room Temperature in 50 mM Tris-HCl Buffer (pH 8.0)

r : mol of Zn^{2+} bound per mol of albumin, Zn_f : concentration of free Zn^{2+} .

This suggests that the serum albumin is species-nonspecifically involved in the fertilization process (Table II).

The effect of albumin concentration on the fertilization rate was further studied. Concentrations of 4 mg/ml and 0.4 mg/ml of albumin were chosen and, under these conditions, fertilization rate was estimated at 0.5, 1.0, 1.5 and 5 h after insemination.

As shown in Table III, it was found that 5 h after the insemination, the fertilization rates observed were the same in both concentrations. However, in a shorter period (e.g. 1 h after the insemination), the fertilization rates were drastically different in both groups. It was obvious that the lower concentration of albumin prolonged the sperm capacitation process.

We already reported the capacitation inhibitory activity of Zn^{2+} .^{7,8)} Therefore, the relationship of Zn^{2+} and albumin in the capacitation phenomenon was investigated. As shown in Table IV, 200 μ M Zn^{2+} completely inhibits the sperm capacitation in the presence of 4 mg/ml of albumin. However, the increase of albumin concentration in the medium competed with the capacitation inhibitory activity of Zn^{2+} , and resulted in the recovery of the fertilization rate.

The state of Zn^{2+} added to the albumin solution was estimated by the equilibrium dialysis method. Fig. 1 shows the Scatchard plots for the interaction of Zn^{2+} with the albumin. The plots were co-linear within the observed area. The free Zn^{2+} in 200 μ M of total zinc added to the

TABLE V. Effects of Albumin on Sperm Penetration of Mouse Ova *in Vitro*

Spermatozoa	Albumin in culture medium	No. of experiments	Total no. of ova examined	Fertilization ^{a)} (%)
Epididymal	4 mg/ml	4	76	69 ± 4
Epididymal	0 mg/ml	4	67	0
Capacitated	0 mg/ml	4	69	77 ± 7

a) Each value represents mean ± S.E. Capacitated spermatozoa were prepared by incubating epididymal spermatozoa in m-KRB for 40 min. Both epididymal and capacitated spermatozoa were washed by centrifugation as described in MATERIALS AND METHODS. Observations were done 90 min after insemination.

medium was determined as $10 \mu\text{M}$ where the mol of Zn^{2+} bound to albumin was 3.3 in Fig. 1. The binding of Zn^{2+} to spermatozoa was studied

by using radio active Zn^{2+} . Out of $10 \mu\text{Ci}$ ($2037730 \pm 5235 \text{ cpm}$) of the $^{65}\text{Zn}^{2+}$ added to the medium, $2015 \pm 326 \text{ cpm}$ and $2866 \pm 490 \text{ cpm}$ were found per 10^6 of epididymal and capacitated spermatozoa, respectively ($n=6$).

The next concern was to check the role of albumin in the sperm penetration stage. The spermatozoa were capacitated in the albumin containing medium and washed by centrifugation after layered on the top of an albumin-free Ficoll containing medium. As shown in Table V, capacitated spermatozoa required no albumin in the medium when penetrating the vicinity of ova.

It was clearly shown in Fig. 2 that the mouse spermatozoa were kept in an epididymal sperm stage (in terms of fertilization ability) during the incubation of the albumin-free medium. The manner of penetration of the preincubated spermatozoa in the albumin-free medium indicates that the spermatozoa began the capacitation process after contact with the albumin.

In a further investigation in the role of albumin in fertilization, the release of fertilization inhibitory substances from sperm was studied. Sperm suspensions were prepared in various kinds of media from which we deleted a substance related to sperm capacitation or acrosome reaction. After 40 min incubation, the supernatants of these sperm suspensions were collected and used as a fertilizing medium after the addi-

FIG. 2. Periodic Observation of Fertilizing of Mouse Ova in Vitro

Each plot represents the fertilization rate of 23–35 ova observed at a certain period.

— ○ —: epididymal spermatozoa,
— △ —: capacitated spermatozoa,
— ● —: preincubated spermatozoa in albumin-free m-KRB.

TABLE VI. Release of Fertilization Inhibitory Substance(s) from Spermatozoa under Various Conditions

Medium in which sperm suspension was prepared	No. of experiments	Total no. of ova examined	Ova penetrated ^{a)} (%)
Control ^{b)}	5	140	61 ± 7
Normal medium	5	133	$13 \pm 3^c)$
Medium without albumin	5	120	77 ± 9
Medium without Ca^{2+}	4	97	$18 \pm 4^c)$
Medium without glucose	5	124	$14 \pm 5^c)$

a) Each value represents the mean $\pm$ S.E. of 4 to 5 experiments

b) Fertilization was performed in normal medium.

c) Significantly different from control: $p < 0.001$.

tion of each deleted substance. In all cases, except albumin deletion, the fertilization rate decreased significantly compared with the control. This indicates that sperm released a fertilization inhibitory substance(s) into the medium. However, in the albumin deleted medium, no fertilization inhibitory activity was found. This clearly demonstrates that a fertilization inhibitory substance(s) was(were) released from sperm themselves and the existence of albumin was essential (Table VI).

DISCUSSION

It has been considered that the albumin plays an important role in the fertilization system. Yanagimachi has succeeded in showing that hamster spermatozoa which had undergone the acrosome-reaction in the presence of bovine follicular fluid acquired the fertilizing ability.⁴⁾ Lui *et al.*, claimed that the albumin is the bovine follicular fluid protein involved in the *in vitro* induction of the hamster sperm acrosome reaction.⁵⁾ However, it remained an open question whether the albumin was necessary in the sperm capacitation process or sperm acrosome reaction process. To examine the proposition, it was necessary to estimate the time of the sperm capacitation process under several conditions by the periodic observation of sperm penetration through the zona pellucida. As shown in Fig. 2, the periodic observation of fertilization enables us to distinguish the difference in penetration time between capacitated and epididymal spermatozoa. Davis *et al.*, in their discussion of the capacitation process, proposed that the albumin absorbs the cholesterol from spermatozoa during capacitation.^{12,13)} On the other hand, Meizel,¹⁴⁾ in his postulated mechanism of acrosome reaction, placed albumin participation near the final stage of the reaction. The results described in this article match with the mechanism proposed by Davis in regard to the following points: 1) the capacitation phenomenon did not proceed until after spermatozoa were incubated in the presence of albumin; 2) the time required for the sperm capacitation was dependent on the con-

centration of albumin.

Furthermore, it was shown in this article that the inhibition of the capacitation process by Zn^{2+} competed with the capacitation inducing activity of albumin (Table IV). Considering that the majority of the Zn^{2+} which was added to the medium was bound to albumin (Fig. 1), we may estimate that one of the capacitation inhibitory mechanisms of Zn^{2+} was based on the binding of the metal ion to albumin preventing the latter from absorbing some factors related to sperm capacitation such as Zn^{2+} , cholesterol and fatty acids from spermatozoa.

The result that the capacitation inhibitory activity of Zn^{2+} is not based upon its absolute concentration, but rather on the relative concentration against albumin, also suggests the above mentioned capacitation inhibitory mechanism. However, Zn^{2+} also may have some direct effect such as the stabilization of the membrane.¹⁵⁾ We postulated the process in the diagram which describes the relationship among the decapacitation factor, albumin and Zn^{2+} in Fig. 3.

The chemical composition of male and female reproductive tract fluids was investigated in detail. According to these studies,¹⁶⁻¹⁸⁾ the female reproductive fluid component is similar to that of serum. However, the testes fluid lacks an albumin fraction. It is likely that the matured spermatozoa contact with high concentration of albumin only after depositing into the female reproductive tracts. Namely, the concentration of 4–32 mg/ml of albumin, which was found to be suitable for *in vitro* fertilization, includes the concentration of 25 mg/ml which was reported by Johnson¹⁹⁾ in human follicular fluid. Moreover, the fact that the increase of albumin concentration is observed in the estrogen dominated rabbit uterus¹⁸⁾ also suggests the direct concern of albumin on the sperm capacitation phenomenon.

The albumin used in the experiments was mainly bovine serum albumin Fr.V (in some cases, mouse serum albumin Fr.V.). In the present paper, we did not attempt a further purification of albumin Fr.V for examination of the

FIG. 3. The Role of the Decapacitation Factor, Zn^{2+} and Albumin in the Sperm Capacitation Phenomenon

The diagram traces the general capacitation process of sperm as they progress through the epididymal, ejaculatory and in vitro stages, as well as clarifying the relationships among decapacitation factor (DF), albumin and Zn^{2+} . Spermatozoa possess many enzymes in their acrosome (described as a "cap"). Enzymes such as hyaluronidase, trypsin-like enzyme and corona penetrating enzyme are believed to be used for the penetration of the investment of ova. The interaction of those enzymes and the decapacitation factor are described elsewhere.^{20,21} The diagram concerns only the capacitation phenomenon, but not the acrosome reaction or sperm penetration stage.

In step A, epididymal sperm are initially depicted as "slumbering on a pillow named DF (decapacitation factor)." Sperm, in their pre-ejaculatory stage, are preserved in the presence of a decapacitation factor which inhibits their fertilizing ability. Ejaculation itself fails to "wake up" the sperm. However, after the sperm enter the female reproductive tract (or under appropriate conditions in vitro), they encounter the capacitation factor "alarm clock" which jars loose the DF. Thus, the sperm acquires a fertilizing capability. Albumin, present in the female reproductive tract, is an important component of the CF (capacitation factor). Albumin is necessary only to "wake up" the sperm. Capacitated sperm can penetrate the ova without an "alarm clock." The time needed for capacitation varies against the concentration of albumin.

Step B summarizes further explorations of the DF's effect on sperm. When capacitated sperm come in contact with a large dose of the DF (the DF may be prepared from seminal plasma or from the sperm themselves), the latter clings to capacitated sperm and returns them to a decapacitated state, characterized by a lower metabolic rate and an inability to fertilize.

The effect of Zn^{2+} is demonstrated in step C. When ejaculated sperm placed in an in vitro environment are exposed to Zn^{2+} , the sperm are prevented from achieving capacitation. Zinc, therefore, functions as a set of "earplugs" which prevent the sperm from responding to the otherwise activating CF.

On the contrary, when previously capacitated sperm are exposed to Zn^{2+} , their fertilizing ability is not extinguished (step D). Zinc, then, has a significant effect only on epididymal sperm, not on capacitated sperm.

Finally, we have considered the capacitation phenomenon that when capacitated sperm are subjected to the DF, they return to a decapacitated stage (step B). Subsequently, when the sperm are restored to an optimal capacitation environment (the female reproductive tract or in vitro), they are successfully recapacitated. Consequently, the DF has been demonstrated as non-toxic (step E),²² since the addition of DF to capacitated sperm does not destroy the fertilizing ability; rather, it renders the sperm temporarily infertile.

capacitation inducing activity. In spite of the declaration of Lui *et al.*,⁵⁾ we still cannot deny the slight possibility that the contaminants in Fr.V might work as a capacitation factor. Therefore, the capacitation inducing activity of albumin is now under investigation from the point of view of determining the active site in the albumin structure.

REFERENCES

- 1) M.C.Chang: Fertilizing capacity of spermatozoa deposited into fallopian tubes, *Nature* (London), **168**, 697–698 (1951).
- 2) C.R.Austin: Observations on the penetration of the sperm into the mammalian egg, *Aust. J. Sci. Res. Ser. B*, **4**, 581–596 (1951).
- 3) J.M.Bedford: Sperm capacitation and fertilization in mammals, *Biol. Reprod. Suppl.*, **2**, 128–158 (1970).
- 4) R.Yanagimachi: *In vitro* acrosome reaction and capacitation of golden hamster spermatozoa by bovine follicular fluid and its fractions, *J. Exp. Zool.*, **170**, 269–280 (1969).
- 5) C.W.Lui, L.E.Cornett and S.Meizel: Identification of the bovine follicular fluid protein involved in the *in vitro* induction of the hamster sperm acrosome reaction, *Biol. Reprod.*, **17**, 34–41 (1977).
- 6) R.Yanagimachi: *In vitro* capacitation of golden hamster spermatozoa by homologous and heterologous blood sera, *Biol. Reprod.*, **3**, 147–153 (1970).
- 7) S.Aonuma, M.Okabe and M.Kawaguchi: The effect of zinc ions on fertilization of mouse ova *in vitro*, *J. Reprod. Fert.*, **53**, 179–183 (1978).
- 8) S.Aonuma, M.Okabe, M.Kawaguchi and Y.Kishi: Zinc effects on mouse spermatozoa and *in vitro* fertilization, *J. Reprod. Fert.*, **63**, 463–466 (1981).
- 9) Y.Toyoda, M.Yokoyama and T.Hoshi: Studies on the fertilization of mouse eggs *in vitro*. II. Effect of *in vitro* preincubation of spermatozoa on time of sperm penetration of mouse eggs *in vitro*, *Jpn. J. Anim. Reprod.*, **16**, 152–157 (1971).
- 10) S.Aonuma, M.Okabe and Y.Kawai: *In vitro* fertilization of mouse ova. I., *J. Pharm. Soc. Japan*, **96**, 1307–1312 (1976).
- 11) S.Aonuma, M.Okabe, Y.Kawai and M.Kawaguchi: The change of solubility properties of zona pellucida to proteases related to fertilizability of mouse ova *in vitro*, *Chem. Pharm. Bull.*, **26**, 405–410 (1978).
- 12) B.K.Davis, R.Byrne and B.Hungund: Studies on the mechanism of capacitation II. Evidence for lipid transfer between plasma membrane of rat sperm and serum albumin during capacitation *in vitro*, *Biochem. Biophys. Acta*, **558**, 257–266 (1979).
- 13) B.K.Davis, R.Byrne and K.Bedigian: Studies on the mechanism of capacitation: Albumin mediated changes in the plasma membrane lipids during *in vitro* incubation of rat sperm cells, *Proc. Natl. Acad. Sci. USA*, **77**, 1546–1550 (1980).
- 14) S.Meizel: The mammalian sperm acrosome reaction: A biochemical approach in "Development in Mammals," ed. by M.H.Johnson, North-Holland, Amsterdam, 1978, pp. 1–62.
- 15) M.Chvapil: New aspects in the biological role of zinc: a stabilizer of macromolecules and biological membranes, *Life Sci.*, **13**, 1041–1049 (1973).
- 16) B.P.Stechell: Secretions of the testis and epididymis, *J. Reprod. Fert.*, **37**, 165–177 (1974).
- 17) R.G.Edwards: Follicular fluid, *J. Reprod. Fert.*, **37**, 189–219 (1974).
- 18) H.M.Beier: Oviducal and uterine fluids, *J. Reprod. Fert.*, **37**, 221–237 (1974).
- 19) M.H.Johnson: Physiological mechanisms for the immunological isolation of spermatozoa in "Advances in Reproductive Physiology," Vol. VI, ed. by M.W.H. Bishop, Logos Press, London, 1973.
- 20) S.Aonuma, M.Okabe, K.Suzuki, K.Matsumoto and Y.Kawai: Studies on sperm capacitation. V. Characterization of decapacitation factor from guinea-pig spermatozoa, *Chem. Pharm. Bull.*, **24**, 907–911 (1976).
- 21) L.J.D.Zaneveld and W.L.Williams: Sperm enzyme that disperses the corona radiata and its inhibition by decapacitation factor, *Biol. Reprod.*, **2**, 363–368 (1970).
- 22) S.Aonuma, T.Mayumi, K.Suzuki, T.Noguchi, M.Iwai and M.Okabe: Studies on Sperm Capacitation I. The relationship between a guinea pig and a sperm capacitation phenomenon, *J. Reprod. Fert.*, **35**, 425–432 (1973).