Citrates-Volume 2 No date

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CITRATES

Volume II

Shinshu I Shi (Journal of the Medical Society of Shinshu), Vol. 9, No. 4, pp. 12 (576)-51 (615).

Pharmacological Studies on Sodium Fluoride in Comparison with Sodium Citrate and Sodium Oxalate.

(Manuscript was received on April 30, 1960).

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Introduction

Fluorine is one of the halogen elements and is widely distributed in nature in the form of fluorspar CaF_2 or cryolite Na_3AlF_6 and in other forms as components of the shell of earth. In addition to its distribution in the mineral world, it also exists mainly as a difficultly-soluble calcium in the animal and plant structures.

According to the descriptions contained in the books written by Heffter (1) and Sollmann (2), Gay-Lussac and Berthold (1803-1805) discovered the existence of fluorine in the regular teeth of man. From the later researches, it has further become clear that fluorine exists most abundantly in the bones and teeth and also exists in small quantities in the skin, hair, nails, blood and milk, etc. and in other intestinal structures.

To examine the physiological and pathological history of this fluorine, Rabuteau (1872) administered sodium fluoride to dogs and rabbits (3) and observed the toxic effects. Following this, Tappeiner (4, 5) and Schultz (6) observed its motion control effect and motion nerve peripheral paralysis action in the case of toads.

Regarding its toxicity, it is reported (according to the books of Heffter, Goodman and Gilman (7)) that the administration of a large amount of the same not only causes gastroenteric inflammation, but also damages various kinds of enzyme activities, causes the scarcity of calcium in blood, prevents the coagulation of blood, lowers the blood pressure, inhibits the heart, causes

epleptic convulsions, promotes the thyroidal function and brings about a retrogressive change in the liver, kidney and other organs.

Sollmann stated that it brings about the cauterization or death of local diaphragms and Siegfrid (8) stated that, even when it is not administered internally, it causes the inflammation of digestive pipe membranes since it damages the tissue respiration of the upper skin of the digestive pipe. In addition, Schultz (6), Schwyzer (9), Greenwood et al. (10), McClure (11), Gottdenker et al. (12), and Sugawa (13) observed a reduction in the (amount of) serum calcium when it is injected in a large quantity. In recent years, Ishimi et al. (14) carried out a determination of serum calcium along with an experiment on the blood pressure of cats and stated that the acute toxic effect of fluorine stems from the removal of calcium (decalcification).

Along with the progress of the chemical industry in recent years, it has been reported that, not infrequently, industrial poisoning is caused when fluorides are pulverized through chemical treatments and absorbed into human bodies through the respiratory organs or digestive organs at plants and factories handling cryolite Na_3AlF_6 and ores containing calcium fluoride CaF_2 and fluorine for the production of aluminum, phosphate fertilizers and in the ceramic industry (15).

Aside from the above, there have been cases involving poisoning caused by the erroneous mixture of the same in the foodstuff as an agent for the prevention of quality deterioration, erroneous drinking of the same or the intake of the same for the purpose of suicide, etc. Minoguchi reported (16) that there was a massive case of fluorine poisoning as a result of the administration of too excessive an amount (by error) of sodium fluoride for the purpose of tooth decay prevention at a certain elementary school within the boundary of Metropolitan Tokyo some years ago.

The amount of the same which causes acute poisoning in human beings is said to be in the range between five and 10 grams, according to Hodge and

Smith (17), and in the range between six and eight milligrams per kilogram, according to Minoguchi. Thus, there is no consensus. The amount of the same causing death is also various; it is in the neighborhood of five grams according to Goodman and Gilman and is assumed to be in the range between eight and 18 grams according to Minoguchi.

In addition to acute toxicity as stated above, chronic poisoning stemming from the in-take of a very small amount of the same which is contained in drinking water for a long period of time has become a problem in recent years. One of the most characteristic changes which are brought about by chronic fluorine poisoning is the change in the teeth (the change being called the spotty teeth or mottled enamel).

Regarding the spotty teeth, von Eager (18) observed a large number of such cases in the volcanic district near Napoli as early as in 1901. He named the phenomenon as Denti di Chjaie. In the United States of America, too, Black and McKay (1910) discovered a similar tooth abnormalcy on one of the branches of the Colorado River (19) and they called it mottled enamel and sought the cause for the same in drinking water. Since that time, similar studies have been carried out in various other countries. Even in our country, Masaki and Mimuro (1931) (22) and Sugawa (1937) (23) discovered the mottled enamel districts in various parts of western Japan for the first time. Later, studies were carried out by a number of researchers and their reports have been published.

The cause of mottled enamel became clear in this manner. Dean et al. (1941) (24) attempted at a reduction in the amount of fluorine contained in drinking water and discovered the fact that there was a reduction in the development of mottled enamel in children's teeth and that the development of decayed teeth among the children was also inhibited. In other words, it became clear that the continuous in-take of drinking water containing an excessive amount of fluorine during the tooth growing period of the children causes the development of mottled enamel in their teeth but that the use of

a small amount of fluorine on a continuous basis serves to prevent decay in the teeth. Since that time, far-reaching studies have been carried out by many researchers in various countries on the effect of fluorides upon the inhibition of decay in the teeth. Thus, excellent results have been obtained in this area.

According to the publications of the United States Public Health Service (25), there was observed a reduction between 50 and 66 per cent in the rate of decay in teeth five years after sodium fluoride was mixed in the waterworks at the concentration of 1.0 p.p.m. Currently, the fluoridation of waterworks has been carried out in 726 districts in the United States for the purpose of preventing tooth decay, with a result that 150,000,000 people are receiving fluoridized drinking water (7). In our country, too, the fluoridation of waterworks was put into effect at the rate of 0.6 p. p. m. in the Yamashina District of Tokyo in 1952. According to an interim report which was prepared five years later, there was shown a marked reduction in the rate of the development of decay in the six-year molar teeth of children in the age range between six and seven, amounting to 65 and 66 per cent of the control group (16).

The prevention of tooth decay by fluorine is not limited to the fluoridation of waterworks as described above. It also involves the internal administration of the same in the form of a mixture in table salt (26) or milk (27) or a chewing gum tablet (28), etc., and an annointment of a fluoride onto the surfaces of teeth (29, 30), the rate of tooth decay prevention being as high as between 50 and 75 per cent.

There are a number of theories regarding the mechanism for the prevention of decay in the teeth by means of fluorine. When fluorine acts upon the teeth during their development stage, a complex salt with calcium is formed and deposited on the surface of the teeth. Pincus (1949) and Volkor (1950) believed that the same plays the role of blocking the route of bacterial infection,

Philips and Swartz (1948-1950) thought that it increased the hardness of the teeth and Cox (1952) (25), Bibby and Kesteren (1940), and Noguchi et al. all believed that the said effect stemmed from the enzyme preventive action of fluorine. In addition to the above, there are other theories including sterilization, etc. In this manner,there is no consensus in theory (31).

Regarding chronic poisoning by fluorine in human beings, in addition to the aforementioned mottled enamel, Hirayama (32) reported a change in the bones and anemia, and Takamori (33) reported disturbances in the myocardium of children and adults based on an examination in some districts where the concentration of fluorine was high (in the range between 1.9 and 4.8 p.p.m.) and confirmed the fact that a similar myocardiac disturbances are developed in his experiment on the chronic poisoning of rabbits. In this manner, damage to living bodies by fluorine poisoning is considerably severe. Sodium fluoride is handled as a poison.

Especially peculiar among the pharmacological effects of fluorine are its effect upon the heart and its enzyme inhibitive effect. Vernon (34,35), De Nitto (36), and Chang (37) et al. observed myocardiac inhibition, Gottdenker et al. (38), Ferrannin (39), and Webb (40), et al. observed a contraction followed by an expansion of the extracted atrium pieces of mammals and Ogiu (41) and Segawa (42) also observed a similar phenomenon in the atrial pieces and further observed a marked inhibitive effect in ECG. In addition, Ogushi et al. (43) also observed a change in rabbit ECG by the feeding of sodium fluoride through the mouth and discussed the inhibitive effect of the same.

On the other hand, Loewi (44) found that the fluorine of a low concentration has a positive inotropic action and attributed it to the reaction with the calcium of the myocardium. Bennet et al. (45) belived that it was due to the reaction with copper ions and Covin and Berman (46) countered by stating that it was due to the enzyme inhibitive action.

Regarding the enzyme inhibitive action of fluorine, on the other hand, there have been a large number of scholars who believe that the acute toxic effect has a close relationship with enzyme inhibition since Artus u. Huber (47) published a report for the first time. Lippmann (48) reported that anaerobic glycolysis in yeast is affected by fluorine, that esterase in phosphatase is inhibited and that its action is similar to the inhibitive action of monoiodoacetic acid.

Moruzzi (49), who injected sodium fluoride in the major arteries of cats, stated that cortical glycolysis was momentarily inhibited and the electric activity of the cerebrum skin portion was lowered. McClure reported that Lipase, esterase and urease are strongly inhibited, that proteolytic and diastatic enzyme are extremely low in sensitivity, that saliva amylase is not inhibited, even when its concentration is high, and that alcoholic fermentation was inhibited.

Sollmann, on the other hand, placed great emphasis upon the enzyme inhibitive action on the assumption that fluorine inhibits the formation of fibrin since the blood coagulation effect of fluorine is not eliminated by the addition of calcium and that the coagulation effect is observed only when thrombine is added. In addition, Fujimoto (5) and Ishimi (51) reported on the inhibition of the flavin enzyme system, and Yonexawa (52) and Hoashi (53, 54) observed the acidic fermentation inhibition of decay tooth bacteria, showing the effect of fluorine upon the enzyme system.

As stated above, there are a large number of basic studies on fluorine. However, very few reports deal with a systematic study of its toxicity and its general pharmacological effects. In addition, there is no consensus of opinion as to the mechanism of its action. In particular, there are doubts as to whether the toxicity and pharmacological action of the fluorides upon the living bodies stem from the mere combination thereof with calcium in the living bodies, thereby reducing the amount of active calcium (Ca") in the living bodies (the effect of removing calcium) or they are related to the special effect of the fluorine ions themselves.

The author selected sodium fluoride which is most widely used as a medicine for the prevention of tooth decay, studied its toxicity and general pharamacological effect, selected sodium citrate and sodium oxalate from among the chemicals having the properties of combining with calcium like fluorine and carried out an experiment on their comparison with sodium fluoride insofar as the pharmacological effects were concerned for the purpose of clarifying the mechanism of the action of fluorine within the living bodies.

By using these three chemicals, the chemical solutions of equivalency to the calculated amounts which were believed to be combined with the same amount of calcium in vitro (the same equivalent concentration) were prepared and the in vitro effect of these chemicals were compared and contrasted.

In this connection, changes in serum calcium, magnesium and inorganic phosphorus were also examined. In addition, an experiment was carried out on the action of sodium fluoride on blood catalase and liver xanthine oxidase and on its action on respiration gas metabolism.

Experimental Method and Experimental Result

Preparation of Experimental Chemicals:

Sodium fluoride NaF, sodium citrate $C_6H_5Na_3O_7.2H_2O$ and sodium oxalate $Na_2C_2O_4$, all pure products of Kanto Chemicals Co. Ltd., were used to make the table salt solutions or Ringer solutions. All chemicals were prepared in such a manner as to assume the same equivalency concentrations corresponding to the same amount of calcium for use in the experiment.

In other words, two molecules of NaF is required for each molecule of $CaCl_2$ in order for F to be united with Ca to produce CaF_2, two molecules of the same is required for every three molecules of $CaCl_2$ for $C_6H_5Na_3O_7.2H_2O$ to produce $Ca_3(C_6H_5O_7)_2.4H_2O$ and further one molecule of the same is required for one molecule of $CaCl_2$ in the case of $Na_2C_2O_4H_2$. When this is converted at the same equivalent concentration, equivalent values are obtained at the concentration ratio of NaF : $C_6H_5Na_3O_2.2H_2O$: $Na_2C_2O_4$ being equal to 1 : 2.4 : 1.6.

If it is possible to explain the pharmacological effects of these three chemicals from the removal of calcium in the living bodies, the three should show the same equivalent actions at the same equivalent concentration. In the author's experiments, the equivalent concentration solutions of all chemicals were prepared at this ratio for the purpose of examination. The pH's of the chemicals used were as follows: one per cent sodium fluoride 7.5, 2.4 per cent sodium citrate 8.2 and 1.6 per cent sodium oxalate 7.3 (all in the table salt aqueous solutions).

The experimental method for each study item will be described in each section.

I. Toxicity and Acute Toxic Symptoms

Experimental Method

Healthy grown male mice whose body weight was around 20 grams were fed mixed feed before they were used in the experiment. Each chemical was dissolved in table salt water and the chemical solutions were prepared to have various concentrations so that the total amount of injections would not exceed 0.5 milliliters per chemical. The observation of the acute toxic symptoms was carried out for a period of 24^h after the injection of the chemicals. The calculation of $LD5_0$ was carried out by using the up-and-down method. The room temperature at the time when the experiment was carried out was in the range between 20 and 23 degrees centigrade.

Experimental Result

a. Sodium Fluoride:

Breathing became rapid and irregular shortly after the injection of 100 milligrams per kilogram and the hair throughout the body stood erect. In the neighborhood of 5^m, the mouth started watering and tears started flowing. In the neighborhood of 10^m, the reaction to external stimuli became high, voluntary motion became active and an excited state was developed. In the neighborhood of 20^m, however, the reaction to the stimuli rather dropped and in the

neighborhood of 30^m, some slight convulsion started but this became more violent in a gradual fashion, with convulsive jumping being observed from time to time. This was followed by the paralysis of the hind limbs, thereby making it impossible to walk and the crawling position was assumed. Around 40^m, four limbs and the trunk of the body were extended and the animal started lying down, and violent convulsion was developed. At the same time, thianose appeared under the nose, and urine discharge and diarrhea were often observed.

In such a state as this, respiratory paralysis was created around 1^h in many cases and the animal died. Violent toxicity similar to that which was described above was shown at 70 milligrams per kilogram and death was brought about in the neighborhood of 1^h in many cases. In those examples which went beyond 1^h, there were many which survived and the symptoms of toxicity were gradually brought back to normal. LD_{50} was in the range between 71.5 and 75.8 milligrams per kilogram (the danger rate being five per cent).

b. Sodium Citrate

In the case of an injection of approximately 240 milligrams per kilogram which corresponds to 100 milligrams per kilogram of sodium fluoride, the outside appearance remained exactly the same as in the regular case, with the number of respirations tending to increase immediately thereafter. In the case of 2.4 grams per kilogram, breathing became markedly rapid and irregular immediately after the injection. The scrotum became swollen, the animal became restless and excited, with a result that it started running around. After the range between 10 and 15^m, hair throughout the body rose (became erect) and the tail became bent like a bow or it was raised upward. Soon thereafter, limb convulsion and the tightening of the "tusks", etc. appeared.

However, many samples withstood several convulsions after $30\text{-}40^m$ and gradually moved back to the original regular state and recovery was achieved in the neighborhood of two to $three^h$.

In the case of an injection of around 3.6 grams per kilogram, these excited states were stronger and quicker in appearing. In the neighborhood of 10^m,

a paralysis of the hind legs, lying down, inverted bow-like extension, the convulsion of four limbs and paralysis took place, followed by respiratory paralysis, and, after 20 to 30^m, death was brought about in many cases. LD_{50} was in the range between 2.67 and 3.09 grams per kilogram. (The danger rate was five per cent).

c. Sodium Oxalate:

No marked toxic symptoms were observed around 160 milligrams per kilogram which was equivalent to 100 milligrams per kilogram of sodium fluoride, with only breathing irregularity and restlessness being observed. In the range between 3 and 4^h, there was observed recovery in many cases. In the case of 800 milligrams per kilogram, breathing became rapid and irregular and restlessness was observed sonn after the injection. Soon thereafter,hair throughout the body rose and the animal soon rounded its back and crouched. Its reactivity to outside stimuli was low, its spontaneous movement became weak and it stopped walking. In the neighborhood of 10^m, it started jumping as if by convulsion and it lay down, repeating clonus and rigid convulsions. In the neighborhood of 15^m, the eye balls stuck out, the four limbs were extended and the animal died after falling into respiratory paralysis. LD_50 was in the range between 345 and 385 milligrams per kilogram (the danger rate was five per cent).

II. Effect upon the Heart

A. Effect upon the Heart in a Living Body

1. Effect on the Heart in the Living Body of a Frog

Experimental Materials and Method Used

"Tonosama" (Master) frogs, whose weight was in the range between 20 and 30 grams, were employed in the experiment. They were paralyzed by injecting 50 milligrams per 10 grams of 10 per cent urethane. After the stoppage of the respiratory movement, the frog was fixed in the backward position, the heart was exposed and the heart movement was drawn on smoked paper by a "haber" in

accordance with the Engelmann method. The chemicals of various concentrations were dissolved in the Ringer solutions and an injection was carried out at less than 0.5 milliliters in the lymphatic sack on the thigh. Observations were continued for a period of 60^m.

Since white turbidity was created when sodium oxalate was dissolved in a Ringer solution, it was shaken well before it was used. The room temperature in this case was in the range between 24 and 28 degrees centigrade.

Experimental Result

a. Sodium fluoride:

With a two x 10^{-6} solution and at 0.005 milligrams per 10 grams, there was only observed a tendency for the number of pulsations to drop. At two x 10^{-5} and 0.005 milligrams per 10 grams, there was observed a tendency for the amplitude to become greater to some extent in the neighborhood of 30m after the injection; however, the number of pulsations tended rather to decline. Mostly no change was observed in tension; however, there were some cases where there was observed a drop in tension. (Refer to Figure 1).

In the case of two x 10^{-5} at 0.005 milligrams per 10 grams, there was observed an increase in amplitude around the range between 30 and 40^m after the injection, with the number of pulsations increasing somewhat. In this case, tension showed a tendency somewhat to decline.

In the case of 0.2 per cent and at 0.5 milligrams per 10 grams, four out of seven examples showed a tendency for the amplitude to be reduced and for the number of pulsations to increase from around the range between five and 10^m after the injection. The three remaining cases showed the opposite tendency, with the amplitude increasing and the number of pulsations decreasing. Tension dropped in all cases. In the case of two per cent at five milligrams per grams s.c. there was observed a marked drop in the ampitude, the number of pulsations and tension around the range betweeen 20 and 30^m, with blocks being observed after $30\text{-}40^m$ (refer to Figure 4).

b. Sodium Citrate:

There was observed no change at a concentration below 4.8×10^{-6}. At 4.8×10^{-5} and 0.012 milligrams per 10 grams, there was observed some slight increase in amplitude and some decline in the number of pulsations from around 40^{m} after the injection. (Refer to Figure 2.) At 4.8×10^{-4} and 0.12 milligrams per 10 grams, there was observed no marked change in amplitude but the number of pulsations alone showed some decrease.

Even at 0.24 per cent and 0.6 milligrams per 10 grams and at 0.48 per cent and 1.2 milligrams per 10 grams, the change observed was approximately the same. At 4.8 per centand at 12 milligrams per 10 grams, there was shown a slight decrease in amplitude and the number of pulsations. (Refer to Figure 5.) Tension was found to drop in all cases above 4.8 and 10^{-5} and 0.012 milligrams per 10 grams and this tendency became all the more marked as the concentration went higher.

c. Sodium Oxalate:

At a concentration below 3.2×10^{-6} and 0.0008 milligrams per 10 grams, s.c., there was observed no change. At 3.2×10^{-5} and 0.008 milligrams per 10 grams, s.c., there was observed some slight increase in amplitude and some slight decrease in the number of pulsations, with no change being observed in tension. (Refer to Figure 3.)

At 3.2×10^{-4} and 0.08 milligrams per 10 grams s.c., there was observed an extremely small decrease in amplitude and an extremely small increase in the number of pulsations. At a concentration and quantity greater than these, tension was found to be lower in all cases. As the concentration and quantity increased, the tendency became stronger.

At 0.32 per cent and 0.8 milligrams per 10 grams s.c., the decline in tension became all the more marked, with blocks being produced in half of the cases 20^{m} afterward. At 3.2 per cent and eight milligrams per 10 grams, there was observed a marked reduction in amplitude and in the number of pulsations in all cases around 15 to 20^{m} and blocks appeared in the neighborhood of 30^{m}.

(Refer to Figure 6.)

(Figure 1 Effect of sodium fluoride on the frog heart in living body.)

(Figure 2 Effect of sodium citrate upon the frog heart in living body.)

(Figure 3 Effect of sodium oxalate on the frog heart in living body.)

(Figure 4 Effect of sodium fluoride on the frog heart in living body.)

(Figure 5 Effect of sodium citrate upon the frog heart of living body.)

(Figure 6 Effect of sodium oxalate on the frog heart of living body.)

2. Effect on the ECG of Rabbits.

Experimental Materials and Method Used

Male grown rabbits, whose weight was in the range between 2.0 and 2.5 kilograms, were paralyzed by an injection of 30 milligrams per kilograms of nembutal into the intestines and they were fixed backwardly to the fixing stand which was insulated. By using a 1/4 injection needle as an induction electrode, it was inserted into and fixed in a certain prescribed position in the four limbs. The recording device used was a total alternating current photographic electrocardiographic type (FAC-2 type) manufactured by Fukuda Electro Company, Ltd. and the secondary induction of the standard limb induction was recorded.

The rabbits were caused to stop eating $12\text{-}16^{h}$ prior to the experiment. Care was exercised so that the room temperature would be maintained in the range between 18 and 20 degrees centigrade. Recording was carried out after the state of pulsation became constant and this was used as the control.

Next, the solution of each chemical (as a table salt aqueous solution) was

ly injected through the concha (vein?) and recording was carried out tely thereafter, 5m thereafter, 10^m thereafter, 30m thereafter, one ^{h} fter and two ^{h} thereafter.

rimental Result

Quiet Injection of Sodium Fluoride:

ost no change was observed in the case of less than five milligrams ogram. In the case of 300 milligrams per kilogram, there was observed ction in the number of heart pulsations in three cases out of four. In nge between 30 and 60^m, some showed a tendency for recovery but there in observed a reduction and the 2^h value was found to have been reduced 0 per minute as compared with the previous value. Almost no change was d in one example. In addition, there were some cases where there was t extension in Q-T in the range between 5^m and 60^m. (Refer to Figure In the case of 50 milligrams per kilogram, there was observed a drop in m the neighborhood of 5^m in three cases out of four. This tendency be- l the more marked as the time passed, complete flatness having been ed 2^h afterward. However, there was no example which showed a complete l.

m around 10^m, both an extension of Q-T and a reduction in the number of ions were observed, with no sign of recovery being observed 2^h after- In one example, however, there was observed a slight increase in the mber of heart pulsations from immediately after to 10^m after, but, the orig- nal value was recovered at 30^m, and a change which was similar to the other amples was shown thereafter. (Refer to Figure 10.)

(Figure 7 Effect of sodium fluoride upon the ECG of bits. a. Control. b. Immediately thereafter.)

g. 8. Effect of sodium citrate upon the ECG of rabbits. a. Control. ediately thereafter.)

Quiet Injection of Sodium Citrate:

the case of 72 milligrams per kilogram, there was observed an increase in mber of heart pulsations from immediately after and this continued until Recovery was made thereafter, with the original value being restored at

2^h. In all cases, there was almost no change in the wave shape. (Refer to Figure 8.) There was observed almost no change when the amount was less than this.

At 120 milligrams per kilogram, the number of pulsations showed an increase in the range between 30 and 40 per minute from immediately after to 10^m, with a decrease being registered thereafter. This trend became most marked at 1^h (registering a drop in the range between 40 and 60 minutes). However, there was almost a complete recovery at 2^h. In the wave shape, there was observed a shortening of the P-Q distance in all cases from immediately after to 5^m, with the P wave being hardly observed in many cases. However, almost complete recovery was achieved at 30^m, and, at the same time, there was observed an extension in Q-T. In any case, recovery was achieved at 2^h. (Reference should be made to Figure 11.)

c. Quiet Injection of Sodium Oxalate:

At 48 milligrams per kilogram, pulsatory irregularity appeared from around 5^m, with the number of heart pulsations increasing slightly. However, there was observed rather a reduction at 30^m, with an extension of Q-T and a drop in S-T being observed. This trend became all themore marked as time passed. (Refer to Figure 9.)

At 72 milligrams per kilogram, respiratory excitement, mouth watering and tear shedding, etc. were developed after the injection in many cases. There was much muscular electric current and there was an expansion of the pupil, with respiration being terminated. As a result, it was almost impossible to carry out a measurement of the ECG.

B. Effect upon Extracted Heart

1. Effect upon the Extracted Heart of a Toad

Experimental Materials and Experimental Method

Toads whose weight ranged from 300 to 500 grams were used. They were fixed in a backward position without anesthesia and their chests were opened to expose the hearts. The hearts were extracted in accordance with the method of

Yagi and they were irrigated with the Ringer solution. At two centimeters H_2O, "cellufin" was applied to the venticles after the state of the heart became constant. By using "Haber", the movement of the heart was drawn on smoked paper. The chemical solutions were caused to flow as the Ringer solutions of various concentrations. After the effects of the chemical solutions were observed, they were exchanged with the Ringer solution once again so as to observe their actions.

Experimental Result

a. Sodium Fluoride

There was observed almost no change by the irrigative flow of a 10^{-8} solution. In the case of the 10^{-7} solution, there was observed a slight reduction in amplitude in one case out of five. Together with the exchange with the Ringer solution, there was the restoration to the original value. In the case of a 10^{-6} solution, there was a slight passing reduction in amplitude but there was a tendency for the same to increase later in three cases out of five. In the remaining two cases, there was observed no change during the irrigative flow. Each time the Ringer solution was exchanged, there was observed a slight increase in amplitude. In one case, the amplitude became constant after there was a slight increase, and it was recovered to the original value together with the exchange with the Ringer solution. At a concentration up to this point, there was observed almost no change both in tension and the number of pulsations. In the case of the 10^{-5} and 2×10^{-5} solutions, there was no marked change in one case out of three, with a slight reduction in amplitude and an increase in the number of pulsations being observed immediately after the irrigative flow of the chemical solutions in two cases, with tension showing a tendency for a slight decrease. In all cases, there was a gradual recovery after the Ringer solution exchange. (Refer to Figure 12.1.)

In the case of the 2×10^{-4} solution, there was observed a marked reduction in amplitude immediately after the irrigative flow of the chemical solutions

in four cases out of five. Among these, blocks appeared during the irrigative flow of chemical solutions in three cases and they appeared each time the Ringer solution was exchanged. In one example, an increase in amplitude appeared passingly; however, the value was soon restored to the original level , and, at the same time, blocks appeared. In many cases, however, recovery was achieved in the neighborhood of 5^m by the exchange of the Ringer solution. (Figure 13.1)

(Figure 9 Effect of sodium oxalate upon the ECG of rabbits. a. Control. b. Immediately thereafter.)

b. Sodium Citrate

There was observed almost no change below the 4.8×10^{-7} solution. In the case of the irrigatibe flow of the 4.8×19^{-6} solution, there was a tendency for a slight increase in amplitude. In the case of the 4.8×10^{-5} solution, the tendency for the amplitude to increase was observed in all cases. One example was especially conspicuous, registering a value which was 2.5 times as great as the previous value after 3^m. However, recovery was achieved along with the exchange with the Ringer solution, the previous value being regained within 10^m. At a concentration up to this, almost no change was observed both in the number of pulsations and in tension. (Refer to Figure 12.2.)

In the irrigative flow of the 2.4×10^{-4} and 4.8×10^{-4} solutions, there was observed a considerably marked decrease in amplitude in both cases; however, recovery was achieved soon after the exchange of the Ringer solution, with an increase being observed around 10^m. Around 20^m, the previous value was almost recovered. (Refer to Figure 13.2). In the case of the 4.8×10^{-3} solution, this tendency was all the more marked. From immediately after the irrigative flow of the chemical solutions, the heart movement brought about an expansive stoppage. The heart movement was gradually started by exchanging the Ringer solution for more than 10 times. With blocks appearing, ten-

sion was observed to be markedly decreased.

c. Sodium Oxalate:

There was observed almost no change at less than 3.2×10^{-7}. In the case of 3.2×10^{-6}, there was observed no change in two cases out of five, with a slight increase in amplitude being observed immediately after the irrigative flow of the chemical solution in three cases. The amplitude increase continued even after the exchange was made to the Ringer solution after 5^{m}. This was recovered to approximately 20^{m} by the exchange of the Ringer solution several times. Even though there was almost no change in the number of pulsations, there was observed a tendency for tension to be slightly reduced.

In the case of the 3.2×10^{-5} solution, there was observed a slight reduction in amplitude in two cases out of three. Simultaneous with the exchange of the Ringer solution, recovery was made, with a tendency for the amplitude to increase being observed thereafter.

A similar amplitude like that of the 3.2×10^{-6} solution was observed in the other example; however, almost no change was observed in tension. (Reference should be made to Figure 12.3.)

In the case of the 3.2×10^{-4} solution, there appeared a marked reduction in amplitude immediately after the irrigative flow, with blocks appearing. Even though there was seen a slight tendency for the number of pulsations to decrease, almost no change was observed in tension. After the Ringer solution exchange, most achieved a quick recovery, with the amplitude once increasing thereafter. Recovery was made by several exchanges of the Ringer solution. (Refer to Figure 13.3).

In the case of 3.2×10^{-3}, the pulsation stopped expansively, tension dropped and no recovery was made even by the exchange of the Ringer solution.

2. Effect on the Extracted Heart of a Frog

Experimental Materials and Method Used

"Tonosama" (Lord) Frogs, which weighed between 22 and 30 grams, were used.

After their heads are cut off and the spines were destroyed, they were fixed backward. The right major artery was tied according to the method of Straub, a "canule" was inserted from the left major artery arc to reach the interior of the venticle and the same was tied. The other arteries and veins were tied together and the heart was extracted.

The pressure of the solution was set at 1.5 centimters H_2O, the Ringer solution was exchanged from time to time, and after the heart movement became constant, "cellufin" was applied to the tip of the heart and its movement was drawn on smoked paper by means of a "haber".

The same chemical solution as in the case of the Yagi system was used. The room temperature was set in the range between 23 and 25 degrees centigrade.

(Figure 10 Effect of sodium fluoride upon rabbit ECG. a. Control. b. Immediately afterward.)

Experimental Result

a. Sodium Fluoride

There was observed absolutely no change in the movement of the heart at less than a two x 10^{-8} solution. In the case of 2 x 10^{-7} solution, there was no change during the irrigation of the chemical solution, with a tendency for the number of pulsations to increase being shown in many cases.

In the case of a two x 10^{-6} solution, there were no changes in three cases out of five, with a passing increase in amplitude being observed in two cases. After 10 to 20^{m} following the exchange of the Ringer solution, there was again a slight increase in the amplitude, with a tendency for the number of pulsations being observed.

In the case of a two x 10^{-5} solution, the action was unstable, with no change being observed in two cases out of eight, and a marked increase in amplitude and an increase in the number of pulsations being observed immediately thereafter in four cases. After 5^{m}, there was observed the recovery of amplitude immediately after the exchange of the Ringer solution. The number

of pulsations returned to the previous value around 20^m.

In two cases, a marked increase in amplitude was shown immediately after the irrigative flow. Of these two, one case showed a continued increase even when the Ringer solution was exchanged more than ten times. In the other case, recovery was achieved $three^m$ after the exchange of the Ringer solution, with a passing block being observed. Almost no change was observed in the number of pulsations and tension. (Refer to Figure 14.1).

(Figure 11 Effect of sodium citrate upon rabbit ECG. a. Control. b. Immediately thereafter.)

In the case of a two x 10^{-4} solution, there was observed a marked reduction in the amplitude and the number of pulsations immediately after the irrigative flow in all cases. The amplitude recovered 5m after the exchange of the Ringer solution, but the number of pulsations did not completely return to the previous value. There was no change in tension, with a slight drop being shown in some cases. At 0.2 per cent, the heart movement develops an expansive stoppage immediately thereafter. When it was cleaned with the Ringer solution several times immediately, recovery was achieved within the range between 10 and 20^m. After this, the amplitude was again reduced and the blocks reappeared in many cases. (Refer to Figure 15.1.)

(Figure 12 Effects of sodium fluoride, sodium citrate and sodium oxalate upon the extracted hearts of toads. R indicates the Ringer solution exchange.)

(Figure 13 Effects of sodium fluoride, sodium citrate and sodium oxalate upon the extracted hearts of toads. R indicates the Ringer solution exchange.)

b. Sodium Citrate

At less than the 4.8 x 10^{-6} solution, there was observed no change. By the irrigative flow of the 4.8 x 10^{-5} solution, there was observed no change in two cases out of five, with an increase in amplitude being observed in

three other cases. Recovery was achieved soon after the exchange of the Ringer solution. There was seen almost no change in the number of pulsations and tension. (Refer to Figure 14.2.)

In the case of 2 4.8 x 10^{-4} solution, there was observed a marked but passing reduction in amplitude immediately thereafter but, around 5^{m}, the previous value was restored in all cases, There was observed no marked change in tension, and recovery was achieved in all cases around 15^{m}. Generally, only a slight increase was observed in the number of pulsations.

In the case of 2 3.8 x 10^{-3} solution, the reduction in amplitude was marked, blocks being observed inthe neighborhood of 3m and the heart movement being stopped in many cases within 5^{m}. No complete recovery was observed even after the exchange of the Ringer solution. Even though there was observed some drop in tension in some cases, no marked change was observed in many other cases. (Refer to Figure 15.2.)

c. Sodium Oxalata

In the case of the irrigative flow of the 3.2 x 10^{-7} solution, the action was not stable, with a slight increase or decrease in the amplitude being shown. In all cases, however, immediate recovery was achieved by the exchange of the Ringer solution.

In the case of the 3.2 x 10^{-6} solution, there was observed a reduction in amplitude in all cases. Immediate recovery was achieved by the exchange of the Ringer solution, with a tendency for it to increase thereafter being observed. Almost no change was observed in the number of pulsations and tension.

In the case of a 3.2 x 10^{-5} solution, there was a more marked reduction in smpoitude, with blocks being observed in many cases. (Refer to Figure 14.3.) In the case of the 3.2 x 10^{-3} solution and 3.2 z 10^{-4} solutions, this tendency became all the more marked, the amplitude being reduced 50 per cent, tension being reduced, the number of pulsations being reduced and blocks being observed in all cases.

There was observed temporary recovery by the exchange of the Ringer solution; however, blocks were shown again and no regular movement was continued. (Refer to Figure 15.3.)

(Figure 14 Effect of sodium fluoride, sodium citrate and sodium oxalate upon the extracted frog hearts (by the Straub method). R indicates the exchange of the Ringer solution.)

(Figure 15 Effects of sodium fluoride, sodium citrate and sodium oxalate upon the extracted frog hearts. R indicates the exchange of the Ringer solution).

3. Effect of Supplementary Addition of Calcium Ions to Various Chemicals.

Experimental Purpose and Experimental Method

As regards the effects upon the extracted frog and extracted toad hearts, since it was known that the solution of approximately 0.02 per cent of soium fluoride, the solution of approximately 0.048 per cent of sodium citrate and the solution of approximately 0.032 per cent of sodium oxalate have an accurate effect of inhibiting the heart, the effects of the supplementary addition of the amount of Ca" which was taken away when these chemicals were dissolved in the Ringer solution upon the extracted hearts of toads were compared with those obtained at the time of the individual irrigative flow of each chemical.

In other words, the calculated amounts by which the three chemicals take away the calcium in the Ringer solution at this concentration were approximately 20 milligrams per deciliter. In order to supplement this as $CaCl_2 \cdot 2H_2O$, approximately 73 milligrams per deciliter was reuiqred. However, $CaCl_2$ has a highly deliquescent character, with a possibility that it may come to have a weight which is less than the weight required theoretically. Therefore, to make certain, approximately 80 milligrams per deciliter was supplementarily added to each solution as $CaCl_2 \cdot 2H_2O$. By using the extracted toad heart as the specimen, the irrigation method of Mr. Yagi was employed.

Experimental Result

a. Sodium Fluoride:

In the case of the Ca" added solution (an NaF 0.02 per cent solution with the addition of $CaCl_2$ 80 milligrams per deciliter), there soon appeared venticular clocks and the number of pulsations decreased, soon after the irrigative flow. In tension, the situation was different from the case of individual irrigation, with almost no change being observed.

Even if the same amount of $CaCl_2$ was supplementarily added to the Ca"-added solution, only an increase in amplitude was observed, with the conductive obstruction being continued. Recovery was achieved soon by the exchange of the Ringer solution 5m later, with the completely normal pulsations being continued thereafter. (Refer to Figure 16.1.)

b. Sodium Citrate:

A passing reduction in amplitude was observed immediately after the irrigation of the 0.048 per cent sodium citrate solution with an addition of 80 milligrams per deciliter of $CaCl_2$; however, recovery was soon achieved and there was observed thereafter a tendency for a slight increase in the same. Even if $CaCl_2$ may be supplementarily added in this case, there was observed no marked change. Along with the exchange of the Ringer solution, the amplitude was restored completely to the time before the irrigation of the chemical solution. (Refer to Figure 16.2.)

c. Sodium Oxalate:

There was observed a passing reduction in amplitude and a decline in tension immediately after the irrigation of the 0.032 per cent sodium oxalate solution with an addition of 80 milligrams per deciliter of $CaCl_2$ as in the case of sodium citrate. However, recovery took place soon thereafter. When $CaCl_2$ was further added thereafter, there was observed a passing increase in tension, with normalcy being restored soon thereafter. (Refer to Figure 16.3.)

III. Effects of Blood Perspiration and Heart Movement of Rabbits.

Experimental Materials and Experimental Method

Male grown rabbits whose weight was around 2.5 kilograms were anesthetized by an intestinal injection of one gram per kilogram of urethane or 40 milligrams per kilogram of nembutal. They were fixed backward and their chests were opened. The heart pulsations were measured by using the myocardiograph of Cushny, and the blood pressure was measured by inserting a "canule" into the neck artery to be connected with a mercury mannometer. As for respiration, a connection was made to a "tampule" of Marey and, at the same time, drawing was carried out on smoked paper.

All of the chemicals were made into table salt solutions and they were quietly injected through the ear vein.

Experimental Result

a. Quiet Injection of Sodium Fluoride:

At an amount of less than five milligrams per kilogram, there was no change in respiration, blood pressure and heart movement. In the range between 20 and 30 milligrams per kilogram, there was shown a passing excitement in breathing, with an increase in amplitude becoming comparatively marked. However, no great change was observed in the number of respirations. Around 10^m, breathing and amplitude becoming rather small. In the neighborhood of 20^m, recovery to the previous value was achieved. Blood pressure started dropping soon after the injection, there being observed a drop of approximately 20 millimeters mercury in the neighborhood of 15^m and recovery being made gradually thereafter. Complete recovery was achieved in the neighborhood of 30^m.

Even though there was shown a passing slight reduction in heart pulsations and amplitude during theinjection, it showed an increase along with the drop of blood pressure. Almost no change was observed in tension. (Refer to Figure 17.)

(Figure 16 Effect of Sodium Fluoride, Sodium Citrate and Sodium Oxalate upon the Toad Extracted Hearts and the Effect of the Additions of Ca". (By the irrigation method of Mr. Yagi.)

1. Sodium fluoride and Ca". 2. Sodium citrate and Ca". 3. Sodium oxalate and Ca". R indicates the exchange of the Ringer solution.)

In the case of 50 milligrams per kilogram i. v., there was observed an increase in the number of respirations and there was a marked increase in amplitude (approximately three times). After this, irregularity set in and the number was rather reduced. In this case, the mouth was often observed to be watering and tears were often observed to be flowing from the eyes. They resorted to violency in many cases. The respiration amplitude returned approximately to the previous value after the range between 20 and 30^m. Even after more than 2^h, recovery was not made to the previous value in some cases. The blood pressure dropped markedly immediately after the injection, with the lowest value being achieved after 10 to 30^m (approximately a 50 millimeters mercury drop), the same being continued to the neighborhood of one^h in many cases.

As regards the heart movement, there was observed a passing reduction in the amplitude soon after the injection. However, recovery took place soon. There was again observed a reduction as the blood pressure dropped. In the neighborhood of 90^m, almost complete recovery was made. Almost no change took place in tension. (Refer to Figure 20.)

In this amount, a violent respiratory excitement was followed by paralysis, with no recovery being made even by artificial respiration, and convulsion, eyeball extrusion, pupil enlargement, mouth-watering and tear flowing, etc. were observed in two cases out of vie. In addition, crying sounds were uttered and death was brought about after the expansive stoppage of the heat movement around 10^m after the injection.

b. Quiet Injection of Sodium Citrate:

There was almost no change by an amount which is less than 20 milligrams per kilogram. At 48 milligrams per kilogram, there was observed a passing excitement in breathing and both the amplitude and the number of respirations increased markedly. After that, there was a temporary reduction. Around 20^m,

however, there was a recovery to the previous value. There was observed an immediate lowering of the blood pressure after the injection (the lowering of approximately 20 millimeters mercury). In the neighborhood of 10^m, the previous value was restored, with a tendency for rise being observed thereafter.

As for the heart movement, there was observed a marked but passing reduction in tension and a decline in amplitude as soon as the injection was carried out. Recovery took place in the neighborhood of 10^m, with a tendency for increase being observed thereafter. Approximately the same change was observed in the case of 72 milligrams per kilogram. (Refer to Figure 18.)

(Figure 17 Effect of sodium fluoride upon the heart movement, blood pressure and respiration of rabbits. 1. Heart movement. 2. Blood pressure. 3. Respiration. 4. Time recording.)

(Figure 18 Effect of sodium citrate upon the heart movement, blood pressure and respiration of rabbits. 1. Heart movement. 2. Blood pressure. 3 Respiration. 4. Time recording.)

At 120 milligrams per kilogram, there was observed a more marked decline in blood pressure (approximately 50 millimeters mercury). The respiratory excitement was also marked and the amplitude increased to five times. The amplitude of the heart movement was markedly reduced and tension was markedly reduced. However, the blood pressure soon recovered, with the previous value being restored in the neighborhood of 30^m and with a tendency for the same to rise thereafter, as in the case of the injection of a small amount. Even though respirations are often terminated thereafter, recovery takes place by two or three artificial respirations. Thereafter, the breathing amplitude increased to four to five times and the number also increased gradually. In the neighborhood of 5^m after the injection, it increased approximately to twice the previous value.

Along with an increase in the number of respirations, the amplitude was rather reduced but almost completely recovery was made in the range between

30 and 40^m. As for the heart movement, there was almost complete recovery in the neighborhood of 30^m as the blood pressure recovered. (Refer to Figure 21.)

When the amount was at this point, mouth watering and tear shedding were observed. In some cases, the animal licked the lips and moved the four legs and became highly violent, with convulsions being observed. All of these were passing, however, with a large number of examples well withstanding such experiences and arrived at the conclusion of the experiment.

When the injection is carried out quickly and rapidly, violent respiratory excitement and paralysis lasted for a long period of time, crying sounds were uttered and the expansive stoppage of the heart was brought about, with the samples dying in some cases.

c. Quite Injection of Sodium Oxalate:

When the amount used was less than eight milligrams per kilogram, there was observed almost no change. At 16 milligrams per kilogram, there was observed a respiratory inhibition after a light passing respiratory excitement. The blood pressure went down gradually. Around 10^m after the injection, there was a decline in the range between 10 and 20 millimeters mercury, lasting for a period of 10 to 20^m thereafter.

(Figure 19 Effect of sodium oxalate upon the heart movement, blood pressure and respiration of rabbits. 1. Heart movement. 3. Blood pressure. 3. Respiration. 4. Time recording.)

(Figure 20 Effect of sodium fluoride upon the heart movement, blood pressure and respiration of rabbits. 1. Heart movement. 2. Blood pressure. 3. Respiration. 4. Time recording.)

As for the heart movement, there was a slight drop in tension and a reduction in amplitude immediately after the injection. Along with the recovery of the blood pressure in 40 to 50^m after the injection, there was observed a recovery, with a tendency for the amplitude to increase being observed thereafter.

(Figure 21 Effect of sodium oxalate upon the heart move-

ment, blood pressure and respiration of rabbits. 1. Heart movement. 2. Blood pressure. 3. Respiration. 4. Time recording.)

(Figure 22 Effect of sodium oxalate upon the heart movement, blood pressure and respiration of rabbits. 1. Heart movement. 2. Blood pressure. 3. Respiration. 4. Time recording.)

If the rate of injections is high in the range between 32 and 48 milligrams per kilogram, there are observed mouth-watering and tear-shedding, with the respirations increasing in number and becoming rapid. The blood pressure comes down aong with the passage of time, finally bringing about respiratory paralysis. The heart movement comes to an expansive stoppage and death is brought about. In this case, no recovery was achieved even when artificial respiration was carried out.

If the injection is carried out slowly in this case (in the range between 3.2 and 4.8 milligrams per kilogram per minute), the blood pressure comes down gradually, reaching the minimum value in the range between 50 and 60^{m} (the drop in the range between 30 and 40 milligrams mercury). In many cases, the previous value was not restored even in the range between 90 and 120^{m}.

The respiratory amplitude increases immediately after, assuming a value which is approximately twice as great, with the number of respirations tending to become smaller. As the amplitude becomes smaller in the range between 20 and 30^{m}, the number tends to increase. The heart movement is inhibited from immediately afterward, with tension being reduced. From 40 to 50^{m} later, there is a gradual increase in amplitude and recovery is made in the neighborhood of 90^{m}. (Reference should be made to Figure 19.)

At 80 milligrams per kilogram, the blood pressure continues to come down. After a respiratory excitement, paralysis is brought about and the heart movement comes to an expansive stoppage to cause death in some cases. (Refer to Figure 22.)

IV. Effect upon the Blood Vessel Center

Experimental Materials and Experimental Method

The experiment was carried out by using the new experimental method used by Kanda et al. (55). This method was employed because it was believed to be superior to the conventional experiment on the pivotal blood vein fuction on the following points: The experimental operations are comparatively simple. Since the animal (rabbit) was to be fixed at the stomach position, it was considered to be comparatively closer to the natural posture. No anesthesia is used. The skin blood vessel (the ear blood vessel) of the upper half of the body is used. The distal blood vessel function should be easily removed. The pure pivotal blood vessel function should be measured without the effect of a change in the heart function.

In other words, an unanesthetized rabbit was fixed to its stomach position. At the base part of the ear, the nerve and both the artery and vein were exposed and separated. Leaving these, the base portion of the ear was cut off by holding it with a forceps. Later, the vein was tied and cut off, a "canule" was inserted into the artery, the Ringer solution from a Mariotte bottle was caused to irrigatively flow to be flowed out of the vein and, when the number of drops became constant, the chemical was injected from the ear vein on the opposite side and the effect was observed. In this case, especial care was exercised so as to protect the nerve, We followed the report of Kanda as to the other points of caution.

Experimental Result

a. Sodium Fluoride:

No marked change took place in the case of a wuite injection of 10 milligrams per kilogram. In the case of a quiet injection of 30 milligrams per kilogram, there was observed an increase of several drops in the neighborhood of 3^m and there was observed an increase of 50 per cent in the neighborhood of 5^m. Recovery was almost achieved around 10^m. In the case of a quiet injection of 50 milligrams per kilogram, there was observed a marked increase in

the number of drops and this increase continued to the neighborhood of 20^m.

b. Sodium Citrate:

When 24 milligrams per kilogram was quietly injected, there was observed no change. In the case of a quiet injection of 72 milligrams, there was observed a passing increase of several drops immediately after the injection, however, recovery was achieved in the neighborhood of $five^m$.

c. Sodium Oxalate:

No change was observed in the case of 16 milligrams per kilogram i.v. In the case of 48 milligrams per kilogram i.v., there was observed a slight increase of several drops in the neighborhood of 5^m after the injection and this lasted to the range between 40 and 50^m. (Reference should be made to Figure 23 for the above descriptions.)

(Figure 23 Effects of sodium fluoride, sodium citrate and sodium oxalate upon the rabbit ear blood vessel irrigation flow (by the Kanda method: the center function of the blood vessel). F: Sodium fluoride 30 milligrams per ilogram i.v., Cit: sodium citrate 72 milligrams per kilogram i.v., Ox: Sodium oxalate 48 milligrams per kilogram i.v. a. Number of, the drops of the irrigation flow solution, per minute. b. Time (minute).)

V. Effect upon the Distal Blood Vessels.

1. Effect upon the Hind Legs of Toads

Experimental Materials and Method

In accordance with the Lawen-Trendelenburg method, the chemical solutions were made into either table salt water or Ringer solutions. One of the Mariotte bottles whose content was 500 milliliters was filled with the Ringer solution or table salt water and the other bottle was filled with the chemical solutions of various concentrations. They were connected by means of a Y-shaped tube and the cock was switched to carry out irrigative flows.

After the number of drops became constant in the range between 20 and 40 drops a minute, switch-over was effected to the chemical solution and irriga-

tion was carried out. After the passage of a certain period of time, the original irrigation solution was restored and the changes, both before and after, were observed and recorded.

Experimental Result

a. Sodium Fluoride:

There was observed almost no effect at less than a 10^{-5} solution, the number of drops was observed to have been reduced to a half by the irrigative flow during the 10^{m} by a 10^{-4} solution and a marked contraction of the blood vessel was observed. However, recovery was achieved as soon as it was switched to the Ringer solution and the regular state was obtained in the neighborhood of 10^{m}. In the case of a 2×10^{-4} solution, the number of drops was reduced to one half in the neighborhood of 10^{m}. When it was switched over to the Ringer solution, there was observed a slight tendency for increasing the number but no recovery to the original value was observed.

In the case of a five $\times 10^{-4}$ solution, this tendency became all the more marked. After 10^{m}, there was observed a reduction in the number of drops in the range between 1/4 and 1/5 and absolutely no trend toward recovery was observed even after it was switched over to the Ringer solution. The reduction continued and there was almost a complete stoppage after 15 to 20^{m}.

b. Sodium Citrate:

There was observed some slight increase in the number of drops from the 4.8×10^{-5} solution. Recovery was achieved immediately after it was switched over to the Ringer solution, with a slight trend for recovery being maintained thereafter in many cases. Even if the concentration was raised and even at the 4.8 per cent solution, there was no marked difference in effect. Only an increase in the number of drops was observed during the irrigation. No recovery was achieved between 30 and 40^{m} after the switching to the Ringer solution.

c. Sodium Oxalate:

In the case of a 3.2×10^{-5} solution, there was observed no definite tendency either for an increase or decrease, with only a slight increase in the number of drops being observed above 3.2×10^{-4}. Even though there were cases where recovery was made to the previous values soon after switching to the Ringer solution, the state was continued in many other cases. In the case of the 3.2 per cent solution, dissolution was incomplete, with a result that, even though the number of drops was reduced, the trend was not definite. (Refer to Figure 24.)

(Figure 24 Effects of sodium fluoride, sodium citrate and sodium oxalate upon the irrigation of the hind legs of toads. F_1: sodium fluoride 0.01 per cent, F_2 sodium fluoride 0.05 per cent, Cit: sodium citrate 0.024 per cent, Ox: sodium oxalate 0.016 per cent. a. Number of drops of the irrigation solution (per minute). b. Time (minute). c. Chemical solution irrigation.)

2. Effect on the Extracted Ear Blood Vessel of Rabbit

Experimental Materials and Experimental Method

The experiment was carried out in conformity with the Krawkow-Pissmski method. As regards the rabbit ear, those which were stored in an ice chamber of four degrees centigrade 12 to 24^h after separation were used. As for the method of irrigation, it was carried out in the same manner as the irrigative flow for the blood vessel of the toad hind legs.

Experimental Result

a. Sodium Fluoride:

In the case of a 10^{-5} solution, there was a slight tendency for the number of drops to increase by several drops but the said tendency was not marked. In the case of a 10^{-4} solution, there was observed a slight reduction of several drops. Even if the irrigation time was prolonged, (30 m), however, the reduction in the number of drops did not increase. Recovery was made immediately upon the exchange of the Ringer solution.

In the case of a 2×10^{-4} solution, there was shown a marked reduction of several drops. This became all the more marked when the time of irrigation was prolonged. After the exchange of the Ringer solution, recovery started in the neighborhood of 30^{m} and, in the neighborhood of 30^{m}, approximately the previous value was restored.

In the case of a five $\times 10^{-4}$ solution, there was a stronger contraction, with no recovery being made even when the Ringer solution was switched on.

At one per cent, however, there was observed no marked change during the irrigation. When an exchange with the Ringer solution was made, there soon was observed a reduction. Thereafter,the value became smaller than the previous value as time passed. At two per cent, there were many cases where there was observed a marked increase of several drops. In some cases, the increase was so large that it was almost impossible to calculate. With the exchange with the Ringer solution, however, the number of drops declined and, in many cases, there was a complete stoppage around 20^{m}.

b. Sodium Citrate:

Some slight reduction in the number of drops was observed around 2.4×10^{-4}. There was no marked effect even in the case of a 4.8×10^{-3} solution with only a slight increase in the number of drops observed. There was rather no effect in the case of a 0.12 per cent solution, with a slight increase having been observed after the exchange of the Ringer solution.

In the case of 2.4 to 4.8 per cent solutions, there was observed an extremely slight reduction in the number of drops. Soon after the exchange of the Ringer solution, there was observed a recovery, a slight increase being observed thereafter.

c. Sodium Oxalate:

In the case of a 3.2×10^{-4} solution, there was observed during irrigation an extremely slight increase in the number of drops. However, recovery was achieved soon after the exchange of the Ringer solution. In the case of a

2.3×10^{-3} solution, a similar effect was observed. Above 1.6 per cent, there was observed a slight reduction in the number of drops. There was observed a recovery by the exchange of the Ringer solution. In a few cases, however, a reduction continued. (Refer to Figure 25).

(Figure 25 . Effects of sodium fluoride, sodium citrate and sodium oxalate upon the rabbit ear blood vessel irrigation. a. Number of drops of the irrigation solution (per minute). b. Time (minute). c. Chemical solution irrigation.)

VI. Effect upon the Urination of Rabbits

Experimental Materials and Method

One hundred (100) millilieters of water was fed to grown male rabbits, whose weight was in the range between two and three kilograms, through the stomach tube. The rabbits were fixed backward by either urethane or nembutal anesthesia and an experiment was carried out in accordance with the ureter "canule" method. In the case where anesthesia was induced by using urethane, the time of so-called urethane diuresis was avoided. After the amount of urea became constant, the chemical solution was slowly injected through the ear vein. The chemical solutions used were the table salt aqueous solutions of two per cent sodium fluoride, 4.8 per cent sodium citrate and 1.6 per cent of sodium oxalate.

Experimental Result

a. Sodium Fluoride;

There was observed no marked effect in the case of five milligrams per kilogram. There was observed a marked increase in the amount of urine in the range between 10 and 20 milligrams per kilogram. In the neighborhood of 10^m, the amount reached six to seven times the amount before the injection, with a decline being observed from around 20^m in a gradual manner. In many cases, however,the previous values were not resotred even at 30^m. In the case of 50 milligrams per kilogram, there was observed a passing increase in the amount of urine amounting to three to four times immediately after the injection. However, this amount was soon reduced and a value which was

smaller than the previous value was observed.

b. Sodium Citrate:

When the amount was smaller than 12 milligrams per kilogram, there was observed almost no effect. In the range between 24 and 48 milligrams per kilogram, however, the amount of urine increased from immediately after the injection. In the range between five and 10^m, the amount reached three to four times the previous value, with the previous value being resotred in the neighborhood of 30^m.

AT 120 milligrams per kilogram, violence was observed immediately after the injection and the amount of urine was passingly reduced to 1/3 to 1/2. In the neighborhood of 10^m, however, respirations became calm and the previous value was restored. The subsequent observation did not show any trend for the amount of urine to increase.

c. Sodium Oxalate:

In the case of 16 milligrams per kilogram, there was observed a slight passing increase in the amount of urine, with the previous value being restored in the range between 10 and 20^m. Thereafter, there was observed a tendency for a slight decrease. In the case of 32 milligrams per kilogram, there was shown an increase of approximately three times in the amount of urine from immediately after the injection, with a mild decline setting in 10 to 20^m after the injection. There was observed a return to the previous value in the neighborhood of 1^h.

In the case of 80 milligrams per kilogram, symptoms which were described in the section dealing with blood pressure and respiration appeared, thereby making it impossible to carry on the measurement. In one example, the amount of urine was reduced to a half and recovery was made around one ^{h} in the case where there was carried out a dropping quiet injection of the Ringer solution during the experiment through the vein in the thigh. (Refer to Figure 26.)

(Figure 26 Rabbit urination test using sodium fluoride, sodium citrate and sodium oxalate. *by the ureter "canule" method). F: Sodium fluoride 20 milligrams i. v., Cit: sodium citrate 48 milligrams per kilogram i.v., Ox: sodium oxalate 32 milligrams per kilogram i.v. a. Number of urine drops ((Translator's Note: the number is not clear but looks like) five minutes). b. Time (minute). c. (Translator's Note: the writing is unclear but looks like) quiet injection.)

VII. Effect upon the Intestinal Tubes of Rabbits.

A. Effect upon Intestinal Tubes in Living Body

Experimental Method

Grown male rabbits, whose weight was in the range between two and three kilograms each, were paralyzed by an injection of 40 milligrams per kilogram of Nembutal into the abdominal cavity, the animal (rabbit) was fixed backward, the abdomens were opened and the movement of the small intestines was drawn on smoked paper by means of a "haber". Since the rabbit become violet during the experiment, care was exercised regarding the instrument for the fixing of the small intestines so that there would be no change in position and tension. The chemical was injected into the vein of the ear.

Experimental Result

a. Quiet Injection of Sodium Fluoride:

At five milligrams per kilogram, there was observed a slight increase in amplitude. At 10 milligrams per kilogram, a marked increase in amplitude and a slight reduction in the number of rhythms continued in the range between 20^m and 30^m. (Refer to Figure 27.) In the case of 30 milligrams per kilogram, there was observed a passing increase in tension immediately after the injection but the previous value was soon restored, with a tendency for a mild increase in tension being observed from around 10^m. There was observed no marked change in amplitude, the number of movements slightly increased and there was observed a slight tendency for amplitude to be slightly

inhibited.

b. Quiet Injection of Sodium Citrate:

There was almost no effect at 12 milligrams per kilogram. At 24 milligrams per kilogram, there was observed a slight passing increase in tension and the number rhythms and a reduction in amplitude; however, recovery was soon made. (Refer to Figure 28.) In the case of 72 milligrams per kilograms, there was observed a passing increase in tension immediate afterward, followed by a passing drop in tension. However, recovery was made soon thereafter. No marked change was observed in amplitude and the number of movements.

c. Quiet Injection of Sodium Oxalate:

There was almost no effect at eight milligrams per kilogram. In the case of a quiet injection of 16 milligrams per kilogram, there were observed a slight passing increase in tension and a trend for an increase in amplitude. However, the number of rhythms was reduced slightly. In all cases, however, recovery to the old value was made in the neighborhood of 15^{m}. (Refer to Figure 29.)

In the case of 48 milligrams per kilogram, there was a rise in tension immediately after the injection, it soon dropped further than the previous value and both amplitude and the number of movements were reduced and no recovery was observed even after the passage of 30^{m}.

B. Effect upon Extracted Intestinal Tubes

Experimental Method

After the rabbits were clubbed to death, their small intestines were immediately extracted and stored in the Tyrode solution. They were fixed backward in the state of untreated living bodies, and their stomachs were opened. The small intestines were cut into pieces ranging from 1.5 to 2.0 centimeters and their movements were drawn on smoked paper in accordance with the Magnus method. The chemicals were prepared as the Tyrode solutions at various con-

centrations and were stored in a constant-temperature vessel. They were exchanged with the Tyrode solutions and the changes were observed. The irrigative flows were carried out at 30 millilieters at all times and the temperature of the solution was set at 38 degrees centigrade.

a. Sodium Fluoride:

There was observed a marked increase in amplitude in the case of the 10^{-6} solution from immediately after the irrigative flow (the increase being about twice), with the increasing trend being observed even after the exchange of the Tyrode solution. Even though tension showed a tendency to increase, there was observed no change in the number of rhythms.

In the case of the 10^{-5} solution, the amplitude was rather inhibited immediately after the irrigative flow, tension dropped passingly and there was a trend for increase along with an increase in amplitude. By the exchange of the Tyrode solution, there was again a reduction in ampoitude and tension. However,these were all passing and recovery was made soon thereafter, followed by an increase in amplitude and a rising trend in tension. (Refer to Figure 30.1.)

In the case of the 10^{-3} solution, the rhythm stopped completely and tension rose markedly. Even after the exchange with the Tyrode solution, the rhythm did not re-start and tension alone continued to rise. (Refer to Figure 30.2.)

b. Sodium Citrate:

In the case of 2.4×10^{-6} and 2.4×10^{-5} solutions, there were observed a passing decline in tension and amplitude immediately afterward; however, recovery was made soon thereafter. Later, there was observed a marked increase in ampoitude and a rise in tension. (Refer to Figure 30.3).

In the case of a 2×10^{-4} solution, the amplitude was reduced to approximately one-half of the previous value. However, recovery was made together with the exchange with the Tyrode solution, with the previous value being restored by the exchange of the Tyrode solution. The previous values were re-

stored in 1^m. However, there was observed a further increase thereafter, showing an increase of more than twice in amplitude. Tension tended to drop slightly during the course of the irrigation in many cases; however, it always went higher after the exchange of the Tyrode solution.

In the case of the 2.4×10^{-3} solution, the rhythms completely stopped and there was observed a drop in tension. The tension further came down when the Tyrode solution was exchanged. However, there was observed a gradual recovery, the movement started again and the tension became greater than the previous value in 3^m and there was an increase in amplitude, too. (Refer to Figure 30.4.)

c. Sodium Oxalate:

In the case of a 1.6×10^{-6} to 1.6×10^{-5} solution, there was observed an marked increase in amplitude and an elevation of tension. In the case of a 1.6×10^{-4} solution, the ampoitude was reduced immediately after the irrigation and a recovery was achieved shortly after the exchange of the Tyrode solution, thereby assuming a value which was greater than the previous value. There was observed a tendency for tension to increase. (Refer to Figure 30.5.) In the case of a 1.6×10^{-3} and 1.6 per cent solution, the rhythm stopped completely and the tension rose markedly. Even when the Tyrode solution was exchanged, no movement was started. In many cases, tension was reduced. (Refer to Figure 30.6.)

VIII. Effects on Variations in the Serum Total Calcium, Dialytic Calcium, Total Magnesium and Inorganic Phosphorus of Rabbits.

Experimental Materials and Experimental Method

Male grown rabbits whose weight was in the neighborhood of 2.5 kilograms were used. They were not given any feed since the night before the experiment. The chemical was quietly injected from the ear vein as a table salt solution.

Collection of Blood:

Two milliliters each was directly taken from the heart by piercing and the one obtained before the injection was used as the control. Twenty^{m}, 1^{h} and h^{h} after the injection, and sometimes even 24^{h} after the injection, the blood collection was made and the blood was put in a centrifugal precipitation pipe with a cock. After coagulation, centrifugal precipitstion was carried out and serum was separated and determined. The room temperature during the experiment was in the range between 17 and 22 degrees centigrade.

Method of Determination:

The serum total calcium (which will hereafter be abbrevated as serum Ca) and the serum total magnesium (which will hereafter be abbreviated as serum Mg) were carried out in accordance with the Yanagisawa Method. (56). According to the Yanagisawa method, serum dialytic calcium (which will hereafter be abbreviated as serum Ca") is extremely restricted in terms of time (three^{m} after the insertion of a reagent to the measurement). Sometimes, the experimental errors were too large. Therefore, the Suzuki's improved method was used.

In other words, 0.2 millilieters of serum was placed in a centrifugal precipitation pipe equipped with a cock and 0.8 milliliters of four per cent ammonium oxalate was added and the mixture was mixed and agitated well for a period of 1^{m}. After the cnetrifugal precipitation was carried out at 1,000 r.p.m. for a period of 1^{m}, 0.5 milliliters of the upper limpid solution was taken to a test tube. By similar operations as the Yanagisawa method, a reagent was added and a measurement was carried out after that.

According to this method, dialytical calcium which has been united with ammonium oxalate is precipitated and remains. Since the calcium which is measured is the calcium of the uniting type, it is possible to obtain an extremely stable value in terms of time.

Serum Ca" was expressed by serum Ca less the amount of cA of the uniting type. Serum inorganic phosphorus (which will hereafter be abbreviated as

serum P) was measured by using the Tokyo Photoelectric Type Photometer 7-A in accordance with the Fiske and Subbarow method (58).

Aside from the individual controls, two milliliters per kilograms of table salt water was quietly injected, and, by using the same blood collection method as for the experimental group, the effect of the amount of blood collected (two millilieters and five milliliters each time) upon the variations in serum Ca, serum Ca", serum Mg and serum P was examined. Table 1 shows the result obtained (Table 1).

(Table 1 Variations in the serum electrolytes by the amount of blood collected. 1. Rabbit No. 2. Amount of blood collected. 3. Time. 4. Total Ca. 5. Dialytical Ca. Total Mg. 7. Inorganic P. 8. Each time. 9. Control. 10. Each time. 11. Control. 12. Each time. 13. Control. 14. Each time. 15. Control. 16. Each time. 17. Control. Legend: The number in the above table shows milligrams per deciliter.)

(Figure 27 Effect of sodium fluoride upon the intestinal pipe in the living rabbit body (per the Trendelenburg method).)

(Figure 28 Effect of sodium citrate upon the intestinal pipe in the living rabbit body.)

(Figure 29 Effect of sodium oxalate upon the intestinal pipe in the living rabbit body.)

(Figure 30 Effects of sodium fluoride, sodium citrate and sodium oxalate upon the extracted intestinal pipes of rabbits (per the Magnus method). 1. Sodium fluoride, 2. Sodium fluoride. 3. Sodium citrate. 4. Sodium citrate. 5. Sodium oxalate. 6. Sodium oxalate.)

When the amount of blood collected each time is two milliliters and from three to four blood collections per 1^h were carried out, there was observed almost no change in the serum electrolyte. In the case of five milliliters, however, serum Ca and serum Ca" were reduced together with the passage of time. On the other hand, serum Mg and serum P showed a tendency for an in-

crease, with clear variations being observed. In the current experiment, therefore, the amount of blood collected was set at two milliliters each.

Experimental Result:

a. Quiet Injection of Sodium Fluoride:

When the amount involved was less than 10 milligrams per kilograms, there were cases where there was observed a reduction in serum Ca. However, there was observed no significant difference. There were no other changes. In the case of 30 milligrams per kilogram i.v., there was clearly a reduction of serum Ca and serum Ca". (Refer to Table 2 and Figure 31.)

If the difference between the control value and the value for each hour in the section dealing with serum Ca in each experiment shown in Table 2 is obtained and if an average of such values is obtained, it will be minus 1.7 milligrams per deciliter for the 20^m values, minus 3.0 milligrams per deciliter for the 1^h values and minus 2.8 milligrams per deciliter for the 4^h value. Among these, the difference for the 1^h values and the 4^h values were the greatest. In each case, the difference was significant.

In the section of serum Ca", there was a parallel reduction, the difference from the 1^h value being minus 1.3 milligrams per deciliter on an average, and minus 1.4 milligrams per deciliter for the 4^h values. These differences were significant.

On the otherhand, there was observed a tendency for the serum Mg and serum P to rise as a general rule. However, the variations are comparatively small. By the same method as above, the difference between the 1^h value of serum Mg and the control value is plus 1.0 milligrams per deciliter on an average which was significant. The difference from thecontrol for the 4^h values was plus 0.7 milligrams per deciliter on an average. This difference was not significant. In addition, the difference from the control for the 1^h values and 4^h values of serum P was plus 0.8 milligrams per deciliter and plus 0.2 milligrams per deciliter respectively, with said difference being no significant

(Refer to Figure 31).

(Table 2 Change in serum electrolyte by sodium fluoride 30 milligrams per kilogram i.v. 1. Rabbit No. 2. Time. 3. Total Ca. 4. Dialytic Ca. 5. Total Mg. 6. Inorganic P. 7. Control. 8. Control. 9. Control. 10. Control. 11. Control. 12. Average value. 13. Control. The numbers shown in the Table show milligrams per deciliter. (-) indicates the absence of an experiment. (The danger rate was five per cent).)

(Table 3 Change in the serum electrolyte by sodium citrate 72 milligrams per kilogram i.v. 1. Rabbit No. 2. Time. 3. Total Ca. 4. Dialytic Ca, 5. Total Mg. 6. Inorganic P. 7. Control. 8. Control. 9. Control. 10. Control. 11. Control. 12. Control. 13. Average value. 14. Control. Legend: The numbers in the Table are milligrams per deciliter.(-) indicates the absence of an experiment. (The danger rate was five per cent).).

(Table 4 Change in the serum electrolyte by sodium oxalate 48 milligrams per kilogram i. v. 1. Rabbit No. 2. Time. 3. Total Ca. 4. Dialytic Ca, 5. Total Mg. 6. Inorganic P. 7. Control. 8. Control. 9. Control. 10. Control. 11. Control. 12. Control. 13. Control. 14. Average value. 15. Control. Legend: The numbers in the Table indicate milligrams per deciliter. (-) indicates the absence of an experiment. (The danger rate was five per cent.))

(Figure 31 Effects of sodium fluoride upon the changes in serum total calcium, dialytical calcium, total magnesium and inorganic phosphorus. (Based on an average of five rabbit examples. 1. Concentration of the serum electrolyte. 2. Time (hours). 3. Before.)

(Figure 32 Effects of sodium citrate upon the changes in the serum total calcium, dialytical calcium, total magnesium and inorganic phosphorus. (based on an average of six rabbit examples. 1. Concentration of the serum electrolyte. 2. Time (hour). 3. Before.)

(Figure 33 Effects of sodium oxalate upon the changes in

serum total calcium, dialytical calcium, total magnesium and inorganic phosphorus. (based on an average of seven rabbit examples.) 1. Concentration of the serum electrolyte. 2. Time (hour). 3. Before.)

b. Quiet In ection of Sodium Citrate:

There was no marked change in the amounts of less than 24 milligrams per deciliter. The result obtained in the case of 72 milligrams per kilogram is shown in Figure 32. In other words, serum Ca is reduced along with time, with the values in the range between 1 and 4^h being the lowest. The difference from the control for the 1^h values is minus 0.9 milligrams per deciliter on an average. It was significant. The difference from the control for the 4^h values was minus 1.1 milligrams per deciliter on an average. However, diffusion was great and no significant difference was obtained. Serum Ca" showed a tendency which was similar to serum Ca. Comparative constancy was maintained and the difference from the control for the hourly values was not significant.

Different from serum Ca, serum Mg showed a slight tendency to increase: but it was not significant. The variation in serum P was also small, with no significant change being observed. (Refer to Table 3, and Figure 32.)

c. Quiet Injection of Sodium Oxalate.

When the amount was less than 16 milligrams per kilogram i.v., there was no marked change except for the fact that there was observed some slight tendency for serum Ca to be reduced. The result obtained at 48 milligrams per kilogram i.v. is shown in Table 4 and Figure 33.

Serum Ca and serum Ca" already started showing a marked drop after 20^m, the 1^h values being the lowest. The difference from the control is minus 3.2 milligrams per deciliter in the case of serum Ca and 1.5 milligrams per deciliter in the case of serum Ca". Even in the case of the 4^h values, the difference from the control was minus 1.7 milligrams per deciliter for serum Ca and minus 1.1 milligrams per deciliter for serum Ca". These differences were all significant.

On the other hand, serum Mg showed a tendency which was opposite, showing

a slight increase. However, some cases like No. 43 showed a slight reduction and the variation was also small. No significant difference was obtained. In addition, the change in serum P was comparatively small. Moreover, the rate of diffusion was high and no accurate result was obtained. (Refer to Table 4 and Figure 33.)

IX. Effects upon the Respiratory Gas Metabolism and Body Temperature of Rats.

Experimental Material and Method Used

Grown male rats whose body weight was in the neighborhood of 200 grams were selected as experimental animals. After they had been fed for a certain period of time, they were subjected to an experiment. As regards the experimental apparatus, a respiratory gas metabolism measuring the device of the Knipping type, as remodeled for small animals by Miyata and Akabane) was used.

As regards the structure of the said apparatus and the method of its use, the instructions of these researchers (59 and 60) were followed and care was likewise exercised to follow their notes in carrying out the experiment.

Efforts were made so that the experimental animals would be accustomed to the apparatus before the experiment, and the amount of O_2 consumed and the amount of CO_2 exhausted during 10^m were measured. For the absorption of CO_2, 15 milliliters of 12 per cent was used. For chasing out CO_2, 10 millimiters of 20 per cent H_2SO_4 was used.

With CO_2/O_2 as the respiration quotient, the amount of basic metabolism was calculated in accordance with the Knipping method. The amounts of basic metabolism and respiratory gas metabolism which were obtained in this manner were recorded as the amount per kilogram per minute and the amount for 24^h per kilogram.

As for the basic metabolism amount, repeated measurements were made on the day of the experiment and the values which were considered to be the most stable were selected and these were compared with the control values before the injection. The feeding of the animals was stopped 15 to 20^h before the

experiment, the room temperature was maintained in the range between 20 and 22 degrees centigrade throughout the experiment and the temperature of the animals chamber was maintained in the neighborhood of 22 degrees centigrade.

In the measurement of body temperature, a bent thermometer having no "stagnant point was used and, immediately after the measurement of respiratory gas metabolism, the measurement was carried out for a period of more than 3^h at a depth of five centimeters in the direct intestines.

The chemicals used were all made into table salt aqueous solutions. By paying attention so that the total amount of the injection would not exceed one milliliter, it was administered into the abdomenalcavity. The experimental results which are shown below are the average values of three examples.

Experimental Result

a. Sodium Fluoride:

In the case of a five milligram per kilogram injection into the abdomenal cavity, there was observed no marked change in the respiratory gas metabolism and body temperature. In the case of 20 milligrams per kilogram, the amount of O_2 1^h after the injection was 18.7 milliliters per minute per kilogram (the value of the control being 22.6 milliters per minute per kilogram), which represents a decline of 17 per cent. Recovery was made thereafter, with the previous value being restored in the third^h. As for the amount of CO_2 discharged, the measured value 1^h after the injection was 16.4 milliliters per minute per kilogram), which represents a decline of five per cent. Thereafter, recovery was made, registering a value of 18.7 milliliters per minute per kilogram at 2^h and 3^h respectively. This represented an increase of nine per cent.

The respiration quotient rose and the body temperature showed a decline of 18 degrees centigrade at 35.5 degrees centigrade after the first h. Recovery was made thereafter, with the regular value being restored on the third hour.

In the case of a 30 milligram per kilogram injection into the abdomenal cavity, the amount of oxygen consumed was measured to be 20.2 milliliters per minute per kilogram at the first hour after the injection (while the control value was 27.0 milliliters per minute per kilogram), which represented a drop of 25 per cent. Recovery was made thereafter on a gradual basis. The second hour value was 23.0 milliliters per minute per kilogram, which showed a 12 per cent drop. On the third hour, however, the previous value was restored.

As regards the amount of CO_2 discharged, the measured value at the first hour after the injection was 17.2 milliliters per minute per kilogram (while the control value was 23.8 milliliters per minute per kilogram), representing a drop of 28 per cent. Recovery was made thereafter, with the second hour value being 20.1 milliliters per minute per kilogram, which represented a drop of 16 per cent. The previous value was almost restored in the third hour. There was observed a tendency for the respiration quotient to drop.

After the first hour, the body temperature was 35.9 degrees centigrade, which represented a marked drop of 2.6 degrees centigrade, with recovery being made thereafter. Even on the third hour, however, there was observed a drop of 0.8 degrees centigrade (Refer to Table 5 and Figure 34.)

b. Sodium Citrate:

In the case of a 48 milligrams per kilogram administration into the abdomenal cavity, the amount of oxygen consumed, as measured at the first hour, was 31.3 milliliters per minute per kilogram (while the control value was 26.6 milliliters per minute per kilogram), which represented an increase of 18 per cent. Recovery was made quickly thereafter, the previous value being almost completely restored at the second hour.

As for the amount of CO_2 discharged,the measured value at the first hour after the injection was 26.6 milliliters per minute per kilogram (while the control value was 23.5 millilieters per minute per kilogram), which represented an increase of 13 per cent. Recovery was made immediately thereafter, the

previous value being almost completely restored at the second hour.

As for the amount of CO_2 discharged, the measured value at the first hour after the injection was 26.6 milliliters per minute per kilogram (while the control value was 23.5 milliliters per minute per kilogram), which represented an increase of 13 per cent. Recovery was made immediately thereafter, the previous value being restored on the second hour. There was observed no marked change in respiration quotient and there was almost no change in the body temperature. (Refer to Table 6 and Figure 35.)

(Table 5 Effects of sodium fluoride, sodium citrate and sodium oxalate upon the rat respiration gas metabolism and body temperature. 1. Sodium fluoride. 2. Sodium fluoride. 3. Sodium citrate. 4. Sodium oxalate 5. Hour. 6. Control. 7. Control. 8. Control. 9. Control. 10. Body temperature. 11. Amount of O_2 consumed. 12. Amount of CO_2 consumed. 13. Respiration quotient. 14. Basic metabolism. 15. Mean value of three examples.)

(Figure 34 Effect of sodium fluoride upon the rat respiration gas metabolism and body temperature. Rat 205 grams. a. Amounts of O_2 and CO_2. b. Before. c. Time (hour). d. Body temperature.)

(Table 6 Effect of sodium fluoride upon blood catalase. 1. Experiment No. 2. Reaction. 3. Measured hour(minute). 4. Control. 5. Control. 6. Control. 7. Control. 8. Control. 9. Control. 10. Average of five examples. Legend: The numbers indicate the O_2-evoltuion.)

c. Sodium Oxalate:

There was almost no marked change in the abdomenal administration of eight milligrams per kilogram. The amount of O_2 consumed at the first hour in the case of 32 milligrams per kilogram was 23.2 milliliters per minute per kilogram (the control value being 25.0 milliliters per minute per kilogram), which represented a drop of seven per cent. Thereafter, there was observed a continued recovery. A drop of 14 per cent was shown in the second hour value of 31.4 milliliters per minute per kilogram. There was a gradual recovery there-

after. The third hour value was 23.2 milliliters per minute per kilogram, which represented a drop of seven per cent.

The measured amount of CO_2 discharged at the first hour was 19.7 milliliters per minute per kilogram (while the control value was 21.5 milliliters per minute per kilogram), representing a decrease of eight per cent and the second hour value was 18.0 milliliters per minute per kilogram, which represented a drop of 16 per cent. Recovery was made thereafter; however, the third hour value was 18.9 milliliters per minute per kilogram, which represented a drop of 12 per cent.

There was observed a tendency for respiration quotient to drop. As for the body temperature, it was 37.0 degrees centigrade after the first hour, which represented a decline of 0.8 degrees centigrade. Recovery was made soon thereafter and the previous value was almost completely restored on the second hour. (Refer to Table 7 and Figure 36.)

(Figure 35 . Effects of sodium citrate upon rat respiration gas metabolism and body temperature. Rat 210 grams. a. Amounts of O_2 and CO_2. b. Before. c. Time (hour). d. Body temperature.)

X. Effect of Sodium Fluoride upon Blood Catalase and Liver Xanthine-Oxidase.

Experimental Materials and Method Used

1. Catalase:

The H_2O decomposition capability of the rabbit blood was measured in accordance with the Warburg method. An enzyme material (the total rabbit blood immediately after the collection was diluted with pure water 100 times) was mixed so that sodium fluoride would be M/300. The temperature of the constant temperature vessel was set at 20 degrees centigrade, the number of agitions was set at 80 per minute and the measurement was carried out for a period of from 30 to 50 m at an interval of 4^m.

2. Xanthine-Oxidase

The rat liver homogenate (containing a structure of 160 milligrams in one cubic centimeter of cold pure water) was used as an enzyme material and Xanthine was used as a substrate. In accordance with the Axelrod-Elvehjem method (61), the measurement was carried out by using the Warburg apparatus. In each experiment, the O_2 absorption (endogenous respiration) in the absence of substrate Xanthine was measured and a correction was carried out at the time when the true Xanthine oxidation rate was sought.

Four reaction systems shown in Figure 38 were prepared. In (1), the endogenous respiration of the liver homogenate was observed. In (2), the effect of sodium fluoride upon the endogenous respiration (E.R.) was observed. In (3) the absorption of O_2 by E. R. and the absorption of O_2 by xanthine oxidation was observed by adding xanthine to (1). In (4), the effect of sodium fluoride on (3) was observed.

(Table 7 a. Experiment No. b. Reaction system measured. c. Measured time (minute). d. Average. Legend: The numbers shown are the amounts of O_2 absorbed (mu)l.)

(Figure 36 Effect of sodium oxalate upon the rat respiration gas metabolism and body temperature. Rat 220 grams. a. Amounts of O_2 and CO_2. b. Before. c. Time (Hour). d. Body temperature.

Experimental Result

1. Effect of Sodium Fluoride on Catalase Activity.

As shown in Figure 37, the H_2O_2 decomposition ability of catalase was markedly lowered by the addition of sodium fluoride in all experimental examples. The rate of the production of O_2 30^m after was approximately 1/4 of the control. (Refer to Figure 37.)

(Figure 37 Effect of Sodium fluoride upon blood catalse. A: Control. B: Sodium fluoride. a. Amount of O_2 produced. b. Time (minute).)

2. Effect of Sodium Fluoride upon Xanthine-Oxidase Activity.

As shown in Figure 38, the result obtained shows that sodium fluoride does not have a constant effect upon the O_2 intake by xanthine oxidation.

In this experiment, further, the O_2 intake by E. R. on the part of the

liver homogenate itself was considerably large, with the difference between this and the intake of O_2 due to xanthine oxidation being small,thereby making it difficult to make a determination of the results obtained. Even though no clear inhibitive action was observed in xanthine-oxidase activity, there was observed in all cases an inhibitive tendency on the part of endogenous respiration (E.R.).

(Figure 38 Effect of sodium fluoride upon xanthine oxidase and endogenous respiration (E.R.). a. Amount of absorption. b. Time (minute).)

It was already pointed out in the introductory section that there is a confrontation of opinion between those scholars (including Schultz, Schwyzer, Loewi and others) who maintained that the action of fluorine in the extracted organ structures and, especially the acute fluorine toxicity in a living body, results from the removal of calcium and the other scholars (including Sollmann, Bannet, Covin and Berman and others) who maintain that it is mainly a result of the enzyme inhibitive action or the peculiar effect of other fluorine ions themselves.

As means for clarifying even a part of these doubts, the present author carried out a comparative experiment on the pharmacological effects of sodium citrate and sodium oxalate which have a strongly uniting character with calcium like fluorine at such a concentration or quantity (the same equivalent concentration) for union with the same amount of calcium in vitro.

The toxicity of sodium citrate is comparatively small. It has been used since olden days as an agent against the coagulation of blood in the case of a blood transfusion and in the blood corpuscle test, etc. Along with the development of surgery and its technique, a large amount of blood is nowadays transfused without much difficulty. As a result, cases involving the toxicity of citric acid have come to be reported.

John et al. (62) examined a number of cases involving acute citric acid toxicity cases which were observed at the time of citric acid blood transfusion and stated that, even though they all were accompanied by a low calcium xymptom but that they were not necessarily saved by a quiet injection of calcium. It also reported that the said toxicity is liable to be caused extremely easily in the case of the rapid and frequent citric acid blood transfusion to patients having liver troubles. The amount of sodium citrate required for causing death is reported in the collection of critical amounts as compiled by Ikeda (63) as being in the range between MLD 0.4 and 1.6 grams in the case of a quiet injection into a rabbit and in the neighborhood of MLD 0.37 grams in the case of a dog.

The toxicity of sodium oxalate is considerably high. According to the textbooks of Sollmann and Goodman and Gilman, when a large amount of this is absorbed into a living body and the membrane is "spoiled" by a strong local action, with a result that nausea, stomach-aches and convulsions, etc. are caused. In addition to various symptoms stemming from low-calcium blood, difficultly-soluble calcium salts block the fine urination tubes, thereby causing pains during urination, no urination or bloody urination, etc.

According to the collection of critical amounts for animals by IKeda, the rabbit hypodermic injection MLD of sodium oxalate is 0.25 grams, the hypoderic injection MLD for rats is 0.4 grams per kilogram and in the range between 0.1 and 0.2 grams per kilogram in the case of quiet injections.

A comparison of acute toxicity and LD_{50} for the mice on the basis of this experimental result shows that, in the case of 100 milligrams per kilogram i. p. of sodium fluoride, a violently excited state is created shortly after the injection, the convulsion being followed by paralysis and respiration being stopped at 30 to 40^{m}, thereby bringing about death.

In the case of the LD_{50} of sodium fluoride, it was in the range between 71.5 and 75.8 milligrams per kilogram (the danger rate being five per cent).

Segawa (42) made sodium fluoride into an aqueous solution, gave an abdomenal injection to both male and female mice, observed toxic symptoms which were similar to those observed in this experiment and calculated the LD_{50} as being approximately 50 milligrams per kilogram. During the rabbit experiment, the author observed few deaths in the case of 50 milligrams per kilogram i.v. and observed no death among the three examples in the case of injections into the abdomenal cavity.

In the case of sodium citrate (240 milligrams per kilogram) of the equivalent concentration as sodium fluoride 100 milligrams per kilogram, there was only observed a slight respiratory excitement. In the case of 2.4 grams per kilogram i. p. in a tenfold amount, there were observed such strong spinal excitations as rapid respirations, irregular respirations, hair rising, tail-raising, and the body elongation like a bow, etc. These were followed by rigidity convulsions. Afterward, there was observed a shift toward paralysis and death was brought about in the range between 30 and 40^{m} in some cases. It is mentioned in this connection that there were many cases where the animals withstood several convulsions, thereby remaining alive. The LD_{50} of sodium citrate was in the range between 2.57 and 3.09 grams per kilogram (the danger rate being five per cent).

In the case of sodium oxalate (160 milligrams per kilogram) i. p., there were observed a slight decrease in the amount of movement and the existence of a quiet state. In the case of 800 milligrams per kilogram, which was five times as large an amount, there was observed hair-rising following irregular respirations. However, such symptoms as the reflective promotion, etc. were not clearly observed. Rather, there was a shift from an active state to a quiet state, rigidity convulsions appearing in the neighborhood of 30^{m}. This was followed by the stoppage of respiration and death. The LD_{50} of sodium oxalate was in the range between 345 and 385 milligrams per kilogram (the danger rate being five per cent).

In other words, toxicity for mice is the strongest in the case of sodium fluoride, followed by sodium oxalate which is in the neighborhood of approximately one-fifth of the same and sodium citrate is less than 1/40 of sodium fluoride. It was sodium citrate in which a marked excitation of the blood vessel center was observed at the time of acute toxicity. In the cases of deaths, such symptoms as elongation like a bow and tail-raising, etc. were observed.

In comparison with the above, sodium fluoride, even when its amount is large, does not show such a marked symptom. In the case of sodium oxalate, there was a quick shift from an excited state to a quiet state.

The excitation of the blood vessel center by sodium citrate can also be clearly observed by injecting an extremely small amount (48 milligrams per kilogram) as viewed from the LD_{50} of the mice. When an injection is carried out slowly by using a considerably large amount, recovery from the excited state takes place in a comparatively short period of time. This is believed to have something to do with the rate of the oxidation of the citric acid which has entered the body.

A comparison made on the basis of the respiration gas metabolism on rats shows the fact that there is no marked change in respiration gas metabolism and in body temperature in the case of five milligrams per kilogram of sodium fluoride, 12 milligram per kilogram of sodium citrate, and eight milligrams of kilogram of sodium citrate.

In the case of an injection of 20 milligrams per kilogram of sodium fluoride, 48 milligrams per kilograms of sodium citrate and 32 milligrams per kilogram of sodium oxalate into the abdomenal cavity, there was shown a peculiar tendency. In the case of sodium fluoride, the amount of O_2 consumed showed the first hour value reduction of 17 per cent and the M amount of CO_2 discharged showed the first hour value reduction of five per cent. Thereafter, however, recovery was shown and the regular state was restored in 3^h.

As for the body temperature, there was observed a marked first hour reduc-

tion of 1.8 degrees centigrade. Thereafter, recovery was made on a gradual basis and the regular state was completely restored in 3^h. In the case of a 30 milligram per kilogram injection, these tendencies appeared more markedly and the holding time was also long.

On the other hand, the amount of O_2 consumed showed the first hour value 18 per cent rise in the case of sodium citrate. The amount of CO_2 discharged showed the first hour value 13 per cent increase. Thereafter, there was observed a recovery, with the second hour value showing a completely recovery to the normal level. In this amount, there was no effect upon the body temperature.

In the case of sodium oxalate, the amount of O_2 consumed showed the first hour value seven per cent reduction and the amount of CO_2 discharged showed a decrease of eight per cent. In the case of the 2^h values thereafter, the amount of O_2 consumed showed a 14 per cent reduction and the amount of CO_2 discharged showed a reduction of 16 per cent. Recovery was observed thereafter. In the case of the 3^h values, the value was lower than the previous value. The body temperature dropped by the first hour value 0.8 degrees centigrade; however, there was observed a recovery thereafter, with the regular value being restored at the second hour.

According to the textbooks of Goodman and Gilman, fluorine ions inhibit the enzyme activity, lowers the anaerobic glycolysis of the extracted organs, inhibits the tissue respirations and inhibits the consumption of oxygen by the muscle and the production of lactic acid. At the same time, the amount of O_2 consumed in the living body is reduced and the amount of CO_2 produced is reduced.

The above is also a fact established by the result that the present author obtained. The administration of 20 milligrams per kilogram of sodium fluoride into the abdomenal cavity inhibited the respiration gas metabolism of rats. In this case, the lowering of the body temperature was observed at all times.

Along with the reduction in the amount of the amount of O_2 consumed, however, it also went down. Since its recovery will also take approximately the same period of time, it is assumed that the production of heat has been reduced as a result of the lowering of metabolic functions by the toxicity of fluorine ions.

Among the three chemicals used, sodium citrate brought about the most violent spinal excitation symptom. Particularly, the respiratory excitement was comparatively small in amount but was observed to a marked degree. In this experiment, the reason why the O_2 consumption amount and the CO_2 discharge amount of rats by 48 milligrams per kilogram i. p., which is much too small as compared with mouse LD_{50} in the case of sodium citrate increased to the first hour values appears to exist in the fact that the general metabolic functions rose passingly and that the oxidation of citric acid inside the body is carried out quickly.

The reduction in the amount of O_2 consumed by sodium oxalate and the amount of CO_2 discharged by sodium oxalate was marked in the second hour values and the holding time was also long. Such a tendency of time is often observed even in other experiments. After this chemical has been absorbed into the body, it becomes a difficultly-soluble calcium salt. As a result, the subsequent decomposition and discharge are delayed or the retrogression of the metabolic function appears gradually because of the tissue toxicity of oxalic acid itself. Moreover, such a state lasts for a long period of time.

The point to which the body temperature declines in parallel with the amount of O_2 consumed is due to the lowering of the production of heat due to the inhibition of or retrogression of the metabolic functions. Its toxicity cannot be ignored.

Aside from the problem of whether the acute fluorine toxicity symptom stems from a reduction in the amount of serum calcium (which will hereafter be abbreviated as serum Ca) or not, an increasingly large number of scholars

recognize the fact that the amount of serum Ca is reduced at the time when a large amount is administered. However, there are divergent views as to the other serum electrolytes which accompany the same.

For example, there are not many reports on serum magnesium (which will hereafter be abbreviated as serum Mg). Sugawa (13) and Bogdanovic (64) reported that the amount of serum inorganic phosphorus (which will hereafter be abbreviated as blood-P) increases but Marcovich (2) reported that the same is reduced.

Regarding sodium citrate and sodium oxalate, Tsai and Hsu (65) reported that in the case of a quiet injection of 50 milligrams per kilogram into a dog, they observed a marked drop in serum Ca and serum P. The values became the lowest in the range between five and 7^h, with an almost complete recovery having been made 24^h later.

Ishimi et al. recently carried out a quiet injection of 50 milligrams per kilogram each of sodium fluoride, sodium citrate and sodium oxalate and examined the change in calcium contained in serum in parallel with the blood pressure test. In all cases, he reported that there was a marked decrease in serum Ca. Regarding 13 patients in which a large amount of blood with an addition of citric acid was observed, Bunker et al. (62) reported that, even though there was observed a marked drop in serum Ca and serum Ca", there was no significant difference in the amount of serum Mg and serum P in this case.

In the measurements of serum Ca which were carried out by a large number of researchers in the past, a considerably large amount of serum was required because of the method of determination involved. Accordingly, the amount of blood collected each time was large. Thus, it was difficult to carry out the collection of blood once and examine the variations thereof in terms of time passed.

The present author carried out the measurement of serum Ca in accordance with the Yanagisawa method. If the amount of blood to be collected each time

was set at five milliliters and several blood collections were carried out each time, the amount of serum Ca would clearly be reduced without the effect of a chemical and the amount of serum P would increase, on the other hand.

Since the Yanagisawa method is characterized in that the amount of the sample was small, it was learned that the effect of anemia would be almost nill, provided that several collections are carried out each time, with the amount of a blood collection being two milliliters each time.

By using the Yanagisawa method, therefore, the effects of these three chemicals upon the serum Ca, serum Ca" and serum Mg of rabbits were examined. At the same time, the variation in the serum P was examined by using the Fiske and Subbarow method. As for the time for blood collection, the rabbit blood pressure experiment was used as a reference,the situation before the administration of the chemical was used as a control and the range between 15 and 20^m after the quiet injection of the chemical where the blood pressure was the lowest and 1^h, 4^h and 24^h where the blood pressure recovered almost completely were selected.

If a comparison is made with 30 milligrams per kilogram of sodium fluoride, 62 milligrams per kilogram of sodium citrate and 48 milligrams per kilogram of sodium oxalate, serum Ca and serum Ca" were found to be the lowest in $1\text{-}2^h$ after the injection in the case of sodium fluoride, with recovery being observed thereafter. Among them, there were some which still showed considerably low values 4^h later. After 24^h, almost the normal state was restored. (Reference should be made to Table 2 and Figures 31.)

In the case of sodium citrate, the rate of a decrease was comparatively small, with the lowest values being observed in the range between 20 and 60^m and a recovery being observed thereafter. At 4^h, there was found almost no differente from the control. (Refer to Table 3 and Figure 31.) Sodium oxalate showed a tendency which was similar to that of sodium fluoride; however, the rate of the reduction of Ca was the most marked.

Serum Ca showed a slight increase along with the passage of time, and showed a correlationship which was opposite to Ca. No accurate result was obtained in the case of the other two.

In the case of serum P, there were many which showed an increase in the 20 to 60^m values after the injection in the case of sodium fluoride, with recovery being observed thereafter but no significant difference being obtained. In the case of sodium citrate, there was observed almost no change. Sodium oxalate tended to show an increase along with the passage of time. In any event, the change was too small to obtain a significant difference.

There have been a large number of researches since olden days regarding the heart functions of sodium fluoride. Its inhibitive action was observed by Vernon (34, 35), who used the heart of a turtle, by De Nitto (36) who used the hearts of mammals, and by Chang (37) who used the atrium specimens of rabbits.

However, Gottdenker u. Rothberger (38) observed an inhibitive effect by using the hearts of frogs and the heart and lung specimens of dogs but pointed out the fact that, in the atriums of rats and rabbits, there appeared transmission obstacles and marked irregular veins between the trunk and atrium, and between the atrium and chamber simultaneously with the effect of increasing the amplitude at the concentration of 0.003M.

In addition, Ferrannini (39) observed an increase in the contraction force of the atrium pieces of rabbits at a concentration in the range between 0.0024 and 0.0072M. Webb (40) examined the relationship between the functions of the myocardium and various metabolic substances and reported that sodium fluoride, at a concentration in the range between 0.005M and 0.05M or at a concentration which completely prevents the desaccharization effect, initially inhibits the amplitude and the number of pulsations, but that, later the number of pulsations tended to stabilize and the amplitude tended to increase.

On the other hand, Loewi (44) reported, in connection with the extracted frog heart, that a positive inotropic action was exercised by sodium fluoride

at a low concentration. He believed that the mechanism involved in this case was that fluorine deprives the myocardium of Ca", thereby producing insoluble CaF_2 to be deposited on the cell membranes of the myocardium and that this affects the myocardium.

Regarding the same effect, Bennet (45) et al. explained that it stems from the reaction between fluorine and copper ions. In addition, Covin and Berman (46) observed the positive inotropic effect of sodium fluoride which was observed in the myocardium specimens of rats from the standpoint of the enzyme inhibitive action and stated that the enzyme oxidation process of APT is not inhibited and that the high energy of phosphorus union would be utilized in the contraction of the myocardium.

Recently, Segawa attempted at an explanation of the mechanism of the effect of sodium fluoride by using the atrium specimens and the nipple muscles in the venticles of rabbits and concluded that the inhibition of the stimulus transmission is the main reason because, in the atrium specimens, sodium fluoride does not bring about irregular veins even if it inhibits the amplitude on a temporary basis and because the drop in the number of pulsations continues even after the recovery of the amplitude.

In the manner described above, the effect of fluorine upon the heart is observed from both the opposing standpoints of inhibition and excitement. Accordingly there are various views as to its mechanism.

When, on the basis of the experimental results the present author obtained by using frog and toad hearts, the effects of sodium fluoride (2×10^{-4}), sodium citrate (4.8×10^{-4}) and sodium oxalate (3.2×10^{-4}) upon the frog hearts in the living body are compared, it is seen that sodium fluoride brings about a slight increase in amplitude and a reduction in the number of pulsations, that almost no change is brought about by sodium citrate and that sodium oxalate brings about a slight decrease in amplitude and an increase in the number of pulsations, thereby already showing the inhibitive

effect. At high concentrations, both sodium fluoride and sodium oxalate show a marked inhibition, with sodium citrate only showing a lowering of tension.

Regarding the extracted frog and toad hearts, the change in amplitude was indeterminate in the case of sodium fluoride, it being impossible to observe only the positive inotropic effect. However, sodium citrate (4.8×10^{-5}) showed a marked increase in amplitude and sodium oxalate (3.2×10^{-5}) showed a tendency which was approximately similar to that of sodium fluoride. When the concentration becomes higher than this, however, there is observed a marked inhibitive effect in all three.

In order to determine whether this inhibitive effect was due tothe lack of Ca" or the effect is exercised in the form of a union with Ca, $CaCl_2$ in an amount equivalent to the removal of Ca was added to each solution of sodium fluoride (2×10^{-4}), sodium citrate (4.8×10^{-4}) and sodium oxalate (3.2×10^{-4}) and a comparison was made with the effects observed at the time of the irrigation by using individual chemicals.

In the case of extracted toad hearts (by the Yagi irrigation method), there was observed a marked reduction in amplitude and the number of pulsations and a transmission inhibition when irrigation is carried out by using sodium fluoride (2×10^{-4}) alone. However, no reduction in amplitude was observed in the case of a $CaCl_3$-added irrigation solution, with a slight tendency for an increase being observed. However,the transmission inhibition appeared at all times. Even if $CaCl_3$ was added supplementarily, amplitude alone was increased with no improvement observed in transmission inhibition.

In the case of sodium citrate (4.8×10^{-4}) on the other hand, the transmission inhibition was not observed in every case even though there was observed a marked inhibitive effect upon the amplitude. When irrigation is carried out by using a $CaCl_3$-added irrigation solution, there is observed a passing inhibition immediately afterward but it disappeared soon, with the recovery being made thereafter and no change whatsoever being detected from

the case of irrigation by using the Ringer solution.

The heart inhibitive effect of sodium oxalate (3.2×10^{-4}) is the most marked and there were cases where there were observed a reduction in amplitude and a transmission inhibition even by using a 3.2×10^{-5} solution. In the case of an irrigation solution which is prepared by adding $CaCl_3$ to a 3.2×10^{-4} solution, there are observed a passing reduction in amplitude and a passing drop in tension, with recovery being observed soon thereafter and the regular state of pulsations being restored thereafter.

Gottdenker and Rothberger showed in the irrigation of extracted frog hearts with calcium fluoride of a concentration which was the same as that of sodium fluoride that there developed a similar transmission inhibition slightly behind the sodium salt and demonstrated in the case where the extracted hearts of mammals were immersed in the Ringer solution that, when the amount of Ca" is around the normal amount of 1/30, the heart stopped in a loosened state but that, in the case of a large amount of sodium fluoride, the heart stopped in the state of a semi-contraction. Based on these facts, they concluded that the reduction of Ca" by sodium fluoride does not immediately bring about a heart inhibitive effect.

Based on the experimental result that the present author obtained, the effect of the supplementary Ca" has no effect whatsoever upon the transmission inhibition which appears by sodium fluoride, clearly different from the cases of sodium citrate and sodium oxalate. It is assumed that the heart inhibitive effect of the sodium fluoride is not a result of the reduction in Ca" alone, but that the peculiar heart effect that fluorine ions have plays an important role. Segawa obtained a similar conclusion by using the atrium speciments of rabbits. It goes without stating, of course, that it is not possible to make a definitive discussion of the mechanism of the heart effect on the basis of these experimental facts alone. Nevertheless, iit is believed that the above will offer means for the clarification of the peculiar effect of fluorine ions

upon the heart.

Kanda et al. (55) published a new method for experimenting on the center of the blood vessel by using the ear blood vessel. When the experimental result present author obtained is compared by using this method, it is observed in the case of the quiet injections of sodium fluoride 30 milligrams per kilogram, sodium citrate 72 milligrams per kilogram, and sodium oxalate 48 milligrams per kilogram that there is only an inhibitive effect upon the center of the blood vessel, with the effect being most marked in the case of sodium fluoride, the effect of sodium citrate being slight and passing and the effect of sodium oxalate being of a lasting nature even though it was slight (it lasted for the neighborhood of 30^{m}). Since the number of experimental cases is small, however, it was not possible to confirm the relationship between the amount of the chemical fed and the degree of expansion. It is the wish of the present author to further continue this study in the future.

Tsunoda et al. (66) examined the effect of sodium fluoride upon the distal blood vessels in accordance with the blood vessel irrigation of the hind legs of toads as well as the extracted rabbit ears, and reported that a marked contraction was observed by the 0.05 per cent solution irrigative flow in all cases. The experimental result obtained by the present author was exactly in agreement with this. We thus confirmed the contraction effect of the distal blood vessels contrary to the blood vessel central action.

When it was more than one per cent, however, the distal blood vessels expanded and there was observed a marked increase in the number of drops at two per cent. When an exchange was made to the Ringer solution in this case, however, the number of drops was soon reduced and a complete termination was brought about in some cases. Aside from the problem of whether this effect is related to the blood pressure effect of sodium fluoride as will be described later, this is merely reported as an experimental fact.

On the other hand, there was observed no marked blood vessel distal effect

of sodium citrate and sodium oxalate. Only an extremely light expansion was observed. The reduction in the number of drops which was observed at the time of the 1.6 per cent irrigative flow of sodium oxalate may not be due to the effect of the chemical but it may be an apparatus phenomenon caused by insoluble calcium oxalate.

One of the features observed at the time of acute fluorine toxicity is the lowering of the blood pressure. This is widely observed by many researchers. However, there is no consensus of opinion as to the mechanism of such an effect. Schultz (6), Schwyzer (9), Greenwood et al. (10), McClure (11) and Ishimi et. al. (14) conclude that it is a result of the tension lowering of the blood vessel walls by the removal of calcium by fluorine. Richardson et al. (67) reported that the lowering of the blood pressure (albeit slightly) appeared when a small amount of sodium fluoride or tin fluoride (five milligrams per kilogram as F) was administered to a dog through the mouth and reported that at this amount there was almost no effect on serum Ca.

Goodman and Gilman described that the effect of fluorine to lower the blood pressure is the result of the myocardiac inhibition and the central nerve inhibition during the movement of the blood vessel which is caused by fluorine.

In the experimental result which was obtained by the present author in the experiments on the blood pressure, respirations and hearts of rabbits, it was learned in the case of a quiet injection of 30 milligrams per kilogram of sodium fluoride that the blood pressure becomes the lowest in the neighborhood of 15^m after the injection, registering a drop of approximately 20 millimeters mercury. Almost complete recovery was made around 30^h. As for the heart movement, there was observed a passing inhibition of amplitude. As the lowering of the blood pressure became marked, it increased and almost complete recovery was made in the neighborhood of 30^m. When the amount of an injection increased, the effect appeared all the more marked and the holding time was likewise prolonged.

In the case of a quiet injection of 72 milligrams per kilogram of sodium citrate which was carried out as a comparison, the blood pressure showed a passing but marked drop, the amplitude of the heart movement was also reduced and tension also showed a marked decrease. Almost complete recovery was made from five to 10^m after the injection. In this case, the respiratory excitement was also marked and the amplitude increased to four times the previous value. Even though it was held down temporarily thereafter, there was again observed an increase in amplitude around 30^m.

In the case of 48 milligrams per kilogram i.v. of sodium oxalate, the blood pressure showed a gradual drop. The time of recovery was also delayed. Its holding time was the longest among the three chemicals. The amplitude of the heart movement was inhibited and showed a gradual recovery from 30 to 40^m afterward.

In this manner, there was similarity insofar as the drop in the blood pressure and the development of respiratory excitement are concerned. However, there are respective characteristics in terms of extent, time and later development, etc.

As for the mechanism for the lowering of the blood pressure, the experimental result which was obtained by the present author on the hearts and the blood circulation system and the results obtained in connection with the examination of the changes in serum Ca will be summarized for observation purposes.

In the case of sodium fluoride, it was not possible to completely eliminate the inhibitive effect of the extracted frog heart (transmission obstacle) by the addition of Ca". On the other hand, Gottdenker et al. reported that the transmission obstacle which was observed by sodium fluoride in connection with the hearts of frogs and turtles and the myocardium of a dog was similarly observed in the case where calcium fluoride of a similar concentration was used.

Even if it may be admitted that it is not possible to directly apply the result of the experiment that the author carried out on the extracted frog heart

to the heart movement of a warm-blooded animal, it appears possible to assume the peculiar effect of fluorine ions upon the hearts of warm-blooded animals.

In the case of a quiet injection of 30 milligrams per kilogram for rabbit ECG, there were observed slow pulsation and, in the case of 50 milligrams per kilogram i.v., there was observed an inhibitive effect stemming from the lowering of S-T and the extension of Q-T.

In the experiment on the action of the center of the blood vessel except for the distal blood vessels, there was already observed an effect of the blood vessel being expanded immediately after the injection in the neighborhood of 30 milligrams per kilogram i.v. In the case of 50 milligrams per kilogram i. v., there was observed a lasting expansion of the blood vessel.

The amount of serums Ca and serum Ca" dropped at 30 milligrams per kilogram i.v. and the lowest value was observed between one and 4^h after the injection. In this instance, the blood pressure of rabbits was the lowest in the neighborhood of 30^m, complete recovery being made in the range between 60 and 90^m. The peaks in both cases did not agree with each other in terms of time.

In view of these experimental facts, it seems that the mechanism of sodium fluoride for the lowering of the pressure cannot be explained simply on the basis of the removal of calcium inside the living body and that the direct cause lies in the strongly inhibitive effect of the blood vessel center and the heart inhibitive effect. It seems that the properties of fluorine ions themselves play an important role in this connection.

On the other hand, sodium citrate has a marked positive inotropic effect at low concentrations upon the frog heart. At high concentration, there is observed an inhibitive effect. As compared with the other two, however, the effect is the least and, even in the inhibitive effect upon the extracted toad heart, the effect of the chemical itself did not show up by the addition of Ca".

In the case of rabbit ECG, there were directly observed frequent pulsations but a recovery was observed soon thereafter. A slight Q-T extension appears

in the range between 30^m and 1^h. All of these are only passing, with recovery being almost completely achieved around 2^h.

As regards the blood vessel center, there is observed a slight passing inhibition at more than 72 milligrams per kilogram i. v., with a result that the blood vessel expands. Recovery is achieved soon thereafter. There is almost no effect upon the distal blood vessels, with a slight tendency for expansion being observed.

As regards the serum Ca and Ca" of rabbits, the values became the lowest in the range between 20 and 60^m after the injection in the case of 72 milligrams per kilogram i. v. There are many cases where recovery was achieved two hours later, thereby indicating the fact that the lasting time of the effect was short.

The effect of inhibiting the frog heart at a high concentration of sodium oxalate is the strongest among the three. However, the mere fact that there was observed almost no effect of sodium oxalate itself upon the extracted toad heart at the time of the irrigation by a Ca"-added solution was an observation which was completely different from the case of sodium fluoride as sodium citrate.

Even in the case of rabbit ECG, the heart inhibitive effect of sodium oxalate is the strongest among the three, with a strongly inhibitive effect being already observed at 48 milligrams per kilogram i. v. The lasting times too, was considerably long. For the blood vessel nerve center, there was observed an inhibitive effect after a latent period following the injection, its lasting time being considerably long, even though its extent was small. There was observed almost no effect upon the distal blood vessels as in the case of sodium citrate.

The amounts of rabbit Ca and Ca" showed a marked drop between 1 and 2^h after the injection of 48 milligrams per kilogram i. v., the degree of the drop being the largest among the three. At this amount, there were few cases

where the recovery was made in blood pressure in the range between 60 and 90m.

From the above-described experimental facts, it can be assumed that the lowering of the blood pressure in the case of sodium citrate and sodium oxalate is participated in by the myocardic inhibition and the inhibition of the blood vessel nerve center. It is, nevertheless, observed that the change in Ca" participates in it as a large factor. This point will have to be examined more carefully in the future.

In all of these three chemicals, both the lowering of the blood pressure and respiratory excitement appeared at the same time. Rather than the chemicals excite the respiratory center, it appears that a reflective character is created by anoxia along with the lowering of the blood pressure. However, the respiratory excitement of sodium citrate always appeared more markedly than in the other two. Judging from the fact that the effect of lowering the pressure is small, it appears that the chemicals themselves have an effect of exciting the respiratory center.

Schwyzer (9) observed a marked increase in the amount of Ca and Cl excreted in urine by the administration of a small amount of fluorine. Ogiu also reported that he had observed an increase in the amount of Cl discharged in urine by administering sodium fluoride to rabbits. Takamori and Kawahara (69) administered 10 milligrams per kilogram and 30 milligrams per kilogram of sodium fluoride to rabbits, observed a marked temporary increase in urine clearance at the initial stage and a marked congestion of blood in the kidney "thread balls", also observed a reduction in protein urine and urine clearance value and observed an increase in the amounts of residual nitrogen of the serum and creatinine.

On the other hand, Kawahara (69) gave a quiet injection of 10 milligrams per kilogram of sodium fluoride and observed an increase in the filtration capacity of the "thread balls" but observed a decrease in GFR and FF in the case of a quiet injection of 30 milligrams per kilogram. Thus, he reported that a pathological observation of the kidney inflammation of the "thread

balls balls" was obtained.

The present author inserted a "canule" into the urination pipe of a rabbit and measured the change in the amountof urine due to the administration of a chemical. In the case of a quiet injection of from 10 to 20 milligrams per kilogram of sodium fluoride, there was observed a marked increase in the amount of urination, ranging from six to seven times, immediately afterward, and the increase lasted from 30 to 40^{m}. In the case of a quiet injection of 50 milligrams per kilogram, however, only a passing increase in urination was observed immediately afterward, and it was soon reduced and completely stopped in some cases.

Regarding the urination effect of the administration of a small amount of fluorine, this effect seems, on the basis of the report published by Kawahara, that it stems from the promotion of the filtration effect of the "thread balls". In view of the fact that fluorine has a strongly enzyme inhibitive effect, if it is admitted that an amount such as this can inhibit the carbon dehydration enzyme, it can be assumed that the urination effect can be created from that. In the experiment that the author carried out this time, no effort was made as to the examination of the function and mechanism. Therefore, a study will have to be carried out on this in the future.

Even in the case of sodium citrate (in the range between 24 and 48 milligrams per kilogram) and sodium oxalate (32 milligrams per kilogram), there was observed a passing increase in the amount of urine. Love (70) reported that the use of these chemicals in the amount of 30 milligrams per kilogram in dogs in the form of a quiet injection increased the amount of the kidney blood. If this is correct, the urination effect caused by these chemicals stems from an increase in the amount of blood circulation stemming from an expansion of the blood vessels in the kidney. However, it was sodium citrate among the three chemicals in which there was observed a marked urination effect, the effects of sodium fluoride and sodium citrate being of a passing

nature and that of sodium oxalate being of a lsting character, albeit in a slight degree.

According to the books written by Heffter and Goodman and Gilman, fluorine promotes the excitability of gastroenteric tubes. Hirata (71) reported that the sensitivity of the extracted intestinal tubes of a rabbit suffering from a chronic fluorine poisoning was greater than that of the extracted intestinal pipes of a regular rabbit.

On the other hand, Salant and Schwartze (72) carried out an experiment on the effects of sodium citrate and sodium oxalate upon the intestinal tubes and reported that a small amount of the same promotes the movement of the small intestines of rabbits but inhibits the movement of the large intestines. He also reported that a large amount of the same inhibited the movements of both the small intestines and the large intestines. Love observed almost the same effect in the case of dogs and reported that it was similar to that of Adrenaline.

According to the experimental result obtained by the author, the movement of small intestines in the living rabbit body is affected in the following manner: When 10 milligrams per kilogram of sodium fluoride, 24 milligrams per kilogram of sodium citrate and 16 milligrams per kilograms of sodium oxalate are used, the sodium fluoride promotes the movement of the small intestines best of all, registering an increase in amplitude. In the case of sodium oxalate and sodium citrate, however, tension rose temporarily but it came down and the amplitude of the movement was reduced to some extent.

In the case of the quiet injections of 30 milligrams per kilogram of sodium fluoride, 72 milligrams per kilogram of sodium citrate and 48 milligrams per kilogram of sodium oxalate, all these three showed a passing elevation of tension immediately after the injection. Sodium fluoride showed a slight decrease in amplitude thereafter, sodium citrate showed an elevation immediately thereafter but showed a decrease afterward. There was almost no change in

amplitude. In the case of sodium oxalate, the number of rhythms as well as amplitude showed a drop.

As regards the extracted rabbit small intestines, the irrigations using a 10^{-6} solution of sodium fluoride, a 2.4×10^{-6} solution of sodium citrate and a 1.6×10^{-6} solution of sodium oxalate showed a marked increase in amplitude in all three cases. In the case of a 10^{-3} solution of sodium fluoride, a 2.4×10^{-3} solution of sodium citrate and a 1.6×10^{-6} solution of sodium oxalate, the rhythms stopped in all three but sodium fluoride increased the tension to a marked extent, the increase continuing even after the exchange with the Tyrode solution.

In the case of sodium citrate, it stopped in the state of tension being lowered, the decrease continuing even after the exchange of the Tyrode solution. However, recovery was achieved soon thereafter, with the rhythms beginning again. In the case of sodium oxalate, it stopped in the state of tension being raised. There was observed a tendency for the same to slightly decline along with the passage of time and it continued further after the exchange of the Tyrode solution.

As stated above, the experimental results for these three chemicals having the effect of removing calcium at the same equivalent concentrations were compared and contrasted. As a result, it was observed that, even though similar tendencies were obserbed in many cases, there was observed a marked difference in the acting time and extent. In particular, completely different observations were made as to the relationship of Ca^{++} with the heart action.

An examination of the reports published by many researchers in the field of the enzyme inhibitive effects of these three chemicals shows that, even though there are many reports on the enzyme inhibitive effect of fluorine as stated in the introductory section, there are no reports stating that sodium oxalate and sodium citrate have an effect of inhibiting the enzyme system. Even the experiment of the present author was carried out on the blood catalase effect of fluorine and the effect on the liver xanthine-oxidase alone

and it is impossible to compare and contrast these three chemicals.

As stated in the individual experimental observations, it is difficult to believe that the effects of the chemicals affecting the functions of complicated living bodies are simply brought about by the temporary change in the serum electrolyte. This is something that can be stated emphatically on the basis of the results obtained in our current experiment. If these chemicals can be compared from the standpoint of the inhibitive action upon the enzyme system in the future, it is believed that it will be possible to understand the peculiarity of fluorine ions. In this study, our efforts were concentrated on the experiment and the observations thereof from the standpoint of general pharmacological effects.

It is expected that the future use of fluorides and sodium fluoride, in particular, which are considered to be most effective as chemicals for the prevention of a tooth decay, will expand rapidly in this field. The author would be most grateful if the clarification of its pharmacological properties would serve as a reference material in terms of basic knowledge.

Summary

The toxicity and general pharmacological effects of sodium fluoride were comparatively observed at the equivalent concentration with sodium citrate and sodium oxalate. As a result, the following conclusions were obtained:

1. As the toxic symptoms created by sodium fluoride upon mice, there was observed an excitement followed by paralysis. In the case of sodium citrate, there was observed a strong center excitation symptom and, in the case of sodium oxalate, there was a shift from the disquiet state to the quiet paralytical state. In the case of LD_{50} by an injection of sodium fluoride into the abdomenal cavity of a mouse, this was in the range between 71.5 and 75.8 milligrams per kilogram, the Ld_{50} of sodium citrate was in the range between 2.67 and 3.90 grams per kilogram and the LD_{50} of sodium oxalate was in the range between 345 and 385 milligrams per kilogram.

2. For the frog heart in the living body, sodium fluoride has an inhibitive effect upon the heart movement above 0.2 per cent and 0.5 milliliters per 10 grams s.c. and it showed a marked reduction in amplitude at two per cent and 0.5 milliliters per 10 grams s.c., with blocks and tension lowering being observed. Approximately the same course was shown by the same equivalent amount of sodium oxalate. In the case of sodium citrate, only the lowering of tension was observed, with almost no change being observed in amplitude and rhythms.

3. For the extracted frog and toad hearts, sodium citrate showed a marked increase in amplitude in the neighborhood of 0.0048 per cent. In the case of the other two, however, no constant tendency was observed. In the case of more than 0.02 per cent of sodium fluoride, there were observed blocks and a marked reduction in amplitude and sodium oxalate showed a similar trend. However, the heart inhibition effect of sodium citrate was the smallest among the three.

4. In the case of irrigation by using a solution which was prepared by adding $CaCl_2$ in an amount calculated for the removal of calcium to a 0.02 per cent sodium fluoride solution, a transmission obstacle appeared slightly behind as compared with the case of irrigation using the chemicals alone, but no reduction in amplitude was observed. Sodium citrate and sodium oxalate of the same equivalent concentration both showed only a passing reduction in amplitude.

5. As for the rabbit ECG, 30 milligrams per kilogram i. v. of sodium fluoride showed no marked change. A similar result was obtained in the case of sodium citrate of the same equivalency. In the case of sodium oxalate, there were observed a marked inhibitive effect such as an S-T drop and a Q-T elongation, etc. Even after the passage of more than 2^h, there was observed no tendency for recovery. In the case of 50 milligrams per kilogram i. v. of sodium fluoride, the lowering of S-T, the flattening of T and the extension

of Q.T, etc. were observed. Sodium citrate showed a slight extension in Q-T, rapid pulsations followed by slow pulsations, with a recovery being observed 2^h later.

6. The blood pressure of rabbits was lowered in all cases by each of these chemicals. The effect was passing in the case of sodium citrate and the effect was slow but lasting in the case of sodium oxalate. Sodium fluoride showed an effect which was in between these two.

7. An excitement was shown in the respiration of rabbits by these chemicals. It was passing but marked in the case of sodium citrate and it was slight in the case of sodium oxalate. Sodium fluoride showed an effect which was in between these two.

8. In the case of the rabbit extracted ear blood vessels and toad hind-leg blood vessels, sodium fluoride showed a marked contraction at 0.05 per cent, and an expansion at two per cent. There was observed almost no effect in the other two.

9. In the experiment on the effect upon the center of the blood vessel, there was observed an expansion of the ear blood vessels of rabbits by all the chemicals. The effect of sodium fluoride was marked and passing, the effect of sodium citrate was light and passing and the effect of sodium oxalate was light but lasting.

10. In the case of extracted intestinal pipes of rabbits (by using the Magnus method), there was observed a marked increase in amplitude at low concentrations by these chemicals. In the case of sodium fluoride, 0.02 per cent stopped the motion and clearly raised tension. Sodium citrate showed a tendency for the tension to become lower. Sodium oxalate showed an effect which was in between these two.

11. As regards the intestinal pipe movement inside the living rabbit body (according to the Trendelenburg method), there was observed a marked increase in amplitude in the case of 10 milligrams per kilogram i. v. of sodium fluoride. In the case of the other two, there was only observed a temporary rise,

followed by a drop, of tension immediately after the injection, with no marked change being observed in amplitude. In the case of a quiet injection of large amounts, the effect of sodium fluoride was marked but those of the other two were slight.

12. In the rabbit urination test using the ureter "canule" method, these chemicals all had the effect of increasing the amount of urine. The effect was most marked in the case of sodium fluoride. The effect of sodium citrate was slight and temporary. The effect of sodium oxalate was lasting.

13. For the rat respiration gas metabolism (by using a respiration gas metabolism measuring apparatus of the Knipping type), sodium flouirde and sodium oxalate showed a decrease in the amounts of O_2 consumed and CO_2 discharged and a lowering of the body temperature. In the case of sodium citrate, on the other hand, there was observed an increase in the amounts of O_2 consumed and CO_2 discharged, with no marked difference being obserbed in body temperature.

14. By a quiet injection of various chemicals, there was observed a reduction in the rabbit serum total calcium and dialytical calcium, the effect of sodium citrate being the smallest. In the case of sodium fluoride, there were simultaneously observed a tendency of slight increase in serum total magnesium and inorganic phosphorus. In the case of the other two, however, no definite trend was observed.

15. Sodium fluoride lowers the H_2O_2 decomposition capability of blood catalase but no apparent inhibitive effect was observed on xanthine-oxidase activity. In all cases, however, there was an inhibitive tendency on endogenous respiration (E. R.).

On the basis of the above-described experimental results, there were observed differences in the pharmacological effects o these chemicals at the same equivalent concentrations, even though they have the same effect of removing calcium. It was observed that the toxicity of sodium fluoride should

be sought only in the removal of calcium. It is believed that the action of fluorine ions themselves has a significance.

The essence of this thesis was presented before the 22nd meeting of the Kanto Chapter of the Pharmacological Society of Japan (on June 19, 1960).

At the end, the author would like to express his deep gratitude to Professor Jiro Akabane who read the manuscript and offered guidance to the author.

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Table 1

1 ウサギ No.	2 採血量	3 時間	4 総Ca	5 透析性 Ca	6 総Mg	7 無機P
No. 2 2.5kg ♂	8 毎回 2 ml	9 対照	14.0	6.1	1.9	6.5
		20m	14.0	6.0	1.8	6.5
		1h	13.9	6.1	1.9	6.7
		4h	14.1	6.2	1.8	6.4
		24h	14.2	6.1	1.8	6.6
No. 3 3.1kg ♂	10 毎回 2 ml	11 対照	15.1	6.5	2.1	6.9
		20m	14.9	6.5	2.0	6.8
		1h	15.0	6.3	1.9	6.9
		4h	15.0	6.2	2.1	6.7
		24h	15.2	6.5	2.2	6.8
No. 4 2.3kg ♂	12 毎回 2 ml	13 対照	10.1	5.1	1.9	6.2
		20m	10.8	5.1	2.1	6.4
		1h	10.5	4.9	2.0	6.4
		4h	9.9	5.0	2.3	6.4
		24h	10.2	5.2	2.2	6.1
No. 5 3.1kg ♂	14 毎回 5 ml	15 対照	13.5	5.8	2.1	6.1
		20m	13.3	5.8	2.0	6.1
		1h	12.1	5.1	2.5	6.5
		2h	11.9	4.5	2.6	7.0
		24h	10.8	4.9	2.7	7.0
No. 6 3.0kg ♂	16 毎回 5 ml	17 対照	11.1	5.4	1.5	5.9
		20m	10.9	5.0	1.6	6.1
		1h	9.9	4.9	1.9	6.5
		2h	9.9	5.1	2.2	6.7
		24h	10.8	4.8	2.2	6.9

Table 2

1 ウサギ No.	2 時間	3 総Ca	4 透析性 Ca	5 総Mg	6 無機P
No. 23 2.6kg ♂	7 対照	12.6	5.9	1.8	6.3
	20m	10.2	5.2	2.8	7.2
	1h	9.6	4.4	3.4	7.2
	4h	9.2	4.4	4.0	7.0
	24h	—	—	—	—
No. 24 2.8kg ♂	8 対照	13.2	5.4	1.5	5.6
	20m	13.2	5.2	1.9	6.2
	1h	10.8	4.3	1.8	8.6
	4h	10.2	4.3	1.6	6.2
	24h	13.3	5.2	1.4	6.0
No. 25 2.85kg ♂	9 対照	10.8	4.0	2.1	4.7
	20m	9.3	3.7	2.9	4.9
	1h	8.2	3.3	3.5	5.2
	4h	9.0	3.4	2.8	4.3
	24h	8.7	4.2	2.9	6.9
No. 31 2.44kg ♂	10 対照	12.4	5.2	1.3	6.1
	20m	10.0	4.0	2.1	7.4
	1h	7.6	2.9	2.3	6.1
	4h	9.0	3.6	1.6	5.0
	24h	—	—	—	—
No. 37 2.5kg ♂	11 対照	12.0	5.8	1.4	6.6
	20m	9.8	4.9	2.5	6.4
	1h	9.9	4.9	2.0	6.4
	4h	9.7	4.1	1.6	7.4
	24h	10.9	5.7	1.1	7.1
12 平均値	13 対照	12.2±1.2	5.3±1.1	1.6±0.5	5.9±1.0
	20m	10.5±2.1	4.6±1.0	2.4±0.6	6.4±1.4
	1h	9.2±1.8	4.0±1.2	2.6±1.1	6.6±1.8
	4h	9.4±0.7	3.9±0.6	2.3±3.0	6.0±1.8
	24h	11.0±7.0	5.0±2.3	1.8±2.9	6.7±1.8

Table 3

1 ウサギ No.	2 時間	3 総Ca	4 透析性 Ca	5 総Mg	6 無機P
No. 21 2.6kg ♂	対照 7	15.2	8.8	0.8	—
	20m	14.2	5.6	1.0	—
	1h	14.4	7.0	0.9	—
	4h	11.0	5.2	1.2	—
	24h	—	—	—	—
No. 22 2.2kg ♂	対照 8	14.6	5.7	2.3	6.0
	20m	13.0	5.5	2.8	6.4
	1h	12.0	5.8	2.7	7.2
	4h	14.8	5.9	2.1	7.5
	24h	—	—	—	—
No. 26 2.9kg ♂	対照 9	10.6	5.1	1.9	5.9
	20m	10.4	5.2	1.9	5.6
	1h	9.8	5.1	2.2	5.7
	4h	7.6	3.7	3.1	6.1
	24h	10.9	5.2	2.3	5.8
No. 27 2.88kg ♂	対照 10	11.0	5.2	2.1	6.1
	20m	10.4	4.3	1.9	5.0
	1h	10.6	4.7	1.9	5.0
	4h	11.2	4.3	2.2	4.6
	24h	10.9	4.8	2.3	4.0
No. 28 3.0kg ♂	対照 11	12.0	5.0	1.9	5.8
	20m	11.4	4.9	1.9	4.2
	1h	11.4	5.0	2.0	4.2
	4h	12.2	5.3	1.8	4.2
	24h	—	—	—	—
No. 29 2.96kg ♂	対照 12	12.0	5.5	0.8	5.9
	20m	12.0	5.3	1.8	6.9
	1h	12.0	5.0	0.8	6.9
	4h	10.0	4.7	1.9	7.1
	24h	11.0	5.1	2.1	6.8
13 平均値	対照 14	12.6±2.2	5.9±1.7	1.6±0.8	5.9±0.2
	20m	11.9±1.7	5.1±0.6	1.9±0.6	5.6±1.5
	1h	11.7±1.8	5.4±1.0	1.8±0.9	5.8±1.7
	4h	11.1±2.7	4.9±0.9	2.1±0.7	5.9±2.0
	24h	10.9±0.2	5.0±0.7	2.2±1.4	5.5±4.3

Table 4

1 ウサギ No.	2 時間	3 総Ca	4 透析性 Ca	5 総Mg	6 無機P
No. 42 3.5kg ♂	対照 7	13.4	5.3	2.0	5.9
	20m	7.0	2.7	1.8	5.9
	1h	7.4	3.4	2.2	5.0
	4h	8.6	4.3	4.0	4.1
	24h	—	—	—	—
No. 43 2.84kg ♂	対照 8	10.9	4.3	1.8	3.4
	20m	7.3	2.5	1.5	3.4
	1h	7.2	2.6	1.2	3.5
	4h	9.2	3.7	1.6	4.2
	24h	9.4	3.9	1.4	6.4
No. 44 2.86kg ♂	対照 9	9.0	4.2	—	—
	20m	6.5	2.8	—	—
	1h	6.5	2.9	—	—
	4h	6.5	2.9	—	—
	24h	7.1	2.7	—	—
No. 45 3.31kg ♂	対照 10	13.8	5.3	1.0	—
	20m	12.9	5.0	—	—
	1h	11.6	4.4	1.4	—
	4h	14.0	5.5	1.0	—
	24h	—	—	—	—
No. 46 3.04kg ♂	対照 11	13.6	5.7	0.9	—
	20m	9.0	3.4	1.2	—
	1h	10.4	4.2	1.4	—
	4h	12.2	4.8	0.9	—
	24h	—	—	—	—
No. 47 2.88kg ♂	対照 12	9.2	4.2	1.2	4.1
	20m	8.0	3.0	2.2	5.0
	1h	8.0	3.0	2.8	4.9
	4h	8.2	2.7	2.4	4.5
	24h	8.2	3.4	2.0	4.9
No. 48 2.0kg ♂	対照 13	10.0	4.6	1.6	5.6
	20m	8.4	4.1	1.2	5.6
	1h	6.4	2.7	2.8	5.4
	4h	9.6	2.2	2.6	5.7
	24h	11.0	4.9	1.4	5.5
14 平均値	対照 15	11.4±2.1	4.8±0.6	1.4±0.5	4.7±2.2
	20m	8.4±2.1	3.4±0.9	1.6±0.6	5.0±2.0
	1h	8.2±2.0	3.3±0.7	1.9±0.8	4.7±1.5
	4h	9.8±2.6	3.7±1.4	2.1±1.5	4.6±1.4
	24h	8.9±2.7	3.7±1.7	1.6±1.1	5.6±2.3

Table 5

	時間	体温 °C	O_2 消費量 ml/min/kg	CO_2 排出量 ml/min/kg	呼吸商	基礎代謝 Cal/24h/kg
弗化ナトリウム 30mg/kg i.p.	対照	38.5	27.0	23.8	0.89	192
	1h	35.9	20.2	17.2	0.86	144
	2h	37.0	23.9	20.1	0.85	169
	3h	37.7	25.4	22.4	0.88	182
弗化ナトリウム 20mg/kg i.p.	対照	37.3	22.6	17.2	0.76	157
	1h	35.5	18.7	16.4	0.88	133
	2h	36.5	20.3	18.7	0.92	145
	3h	37.5	33.5	18.7	0.80	164
クエン酸ナトリウム 48mg/kg i.p.	対照	38.1	26.6	23.5	0.88	189
	1h	38.1	31.3	26.6	0.85	222
	2h	38.0	25.8	23.5	0.90	185
	3h	38.0	26.6	23.5	0.88	189
蓚酸ナトリウム 32mg/kg i.p.	対照	37.8	25.0	21.5	0.86	178
	1h	37.0	23.2	19.7	0.85	165
	2h	37.5	21.4	18.0	0.84	152
	3h	37.9	23.2	18.9	0.82	164

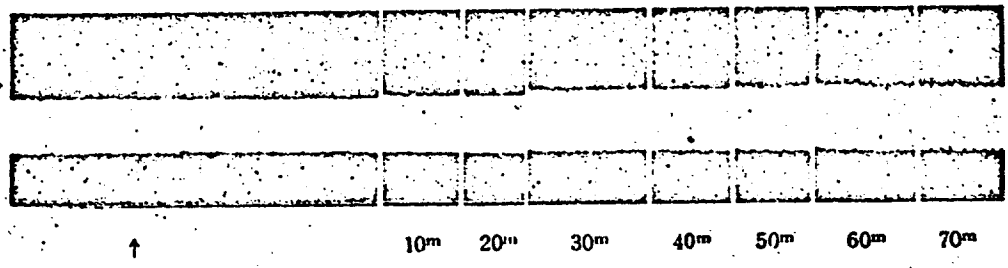
3 例平均値

Table 6

実験番号	反応	測定時間 (分) 2	6	10	14	18	22	26	30	35	40
1	対照	69.0	134.0	188.0	231.0	266.0	296.0	317.0	334.0	350.0	361.0
	NaF	11.2	24.3	37.0	48.9	61.0	71.0	81.5	91.0	101.0	110.0
2	対照	38.2	93.0	131.0	156.0	198.0	223.0	245.0	264.0	286.0	304.0
	NaF	6.85	13.8	20.0	25.8	32.5	38.0	43.8	48.0	58.0	62.1
3	対照	15.9	36.8	53.7	65.5	76.5	84.5	95.5	102.0	108.0	112.0
	NaF	3.95	10.2	19.0	22.9	28.8	34.0	40.4	45.6	50.5	57.0
4	対照	41.2	92.0	131.0	167.0	198.0	226.0	249.0	270.0	294.0	309.0
	NaF	4.05	10.5	17.6	23.6	29.8	35.6	40.8	46.5	53.0	58.5
5	対照	63.4	125.0	177.0	220.0	254.0	277.0	300.0	313.0	327.0	334.0
	NaF	7.0	16.6	30.3	43.6	57.0	65.4	79.5	88.6	99.5	113.0
5例平均	対照	45.7	95.2	136.1	167.9	198.4	211.3	241.3	256.6	273.0	284.0
	NaF	6.6	15.1	24.8	33.0	41.8	48.8	57.2	63.9	72.4	80.1

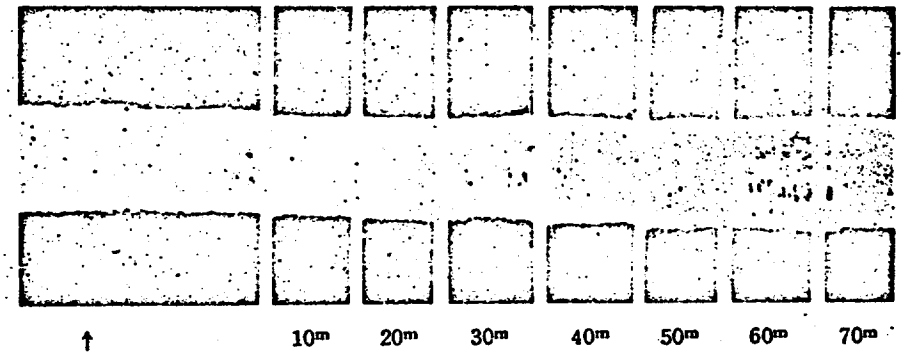
Table 7

a 実験番号	b 測定反応系	c 測定時間 (分) 20	40	60	80	100	120	140	160	180
1	E.R.	29.8	48.8	59.5	65.5	76.1	89.3	98.0	101.0	115.0
	E.R.+NaF	22.0	34.6	47.5	53.4	56.7	60.3	61.8	61.7	63.0
	E.R.+Xanth.	43.7	64.2	73.5	85.0	95.5	100.0	121.1	123.0	138.0
	E.R.+Xanth.+NaF	29.6	40.2	49.3	53.8	57.3	60.4	63.4	65.3	66.1
2	E.R.	29.3	47.7	55.4	62.4	63.9	74.0	83.0	94.6	112.0
	E.R.+NaF	35.8	50.2	59.3	65.7	69.0	71.8	74.5	79.2	82.6
	E.R.+Xanth.	45.7	63.9	70.0	78.5	84.0	90.1	100.0	111.0	123.0
	E.R.+Xanth.+NaF	32.0	45.0	51.9	58.9	62.3	64.5	68.1	70.7	71.6
3	E.R.	27.0	39.2	48.5	57.0	61.0	67.6	73.0	81.5	88.5
	E.R.+NaF	20.9	28.7	34.0	42.1	45.6	49.8	50.6	52.3	54.1
	E.R.+Xanth.	28.4	39.6	44.3	53.4	57.0	61.0	66.5	75.0	82.5
	E.R +Xanth.+NaF	27.4	33.6	39.8	44.3	47.8	49.9	53.0	55.6	57.8
d 平均	E.R.	28.7	45.2	54.5	61.6	67.0	77.0	84.7	92.4	105.2
	E.R.+NaF	26.2	37.8	46.9	47.0	57.1	60.6	62.3	64.4	66.6
	E.R.+Xanth	39.0	55.9	62.6	72.3	78.8	83.7	95.8	103.0	114.5
	E.R.+Xanth.+NaF	29.7	39.6	47.0	52.3	55.8	58.3	61.5	63.9	65.2



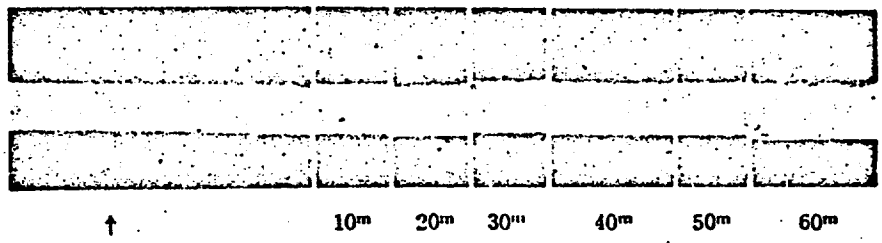
NaF 0.002, %0.005mg/10g

Figure 1



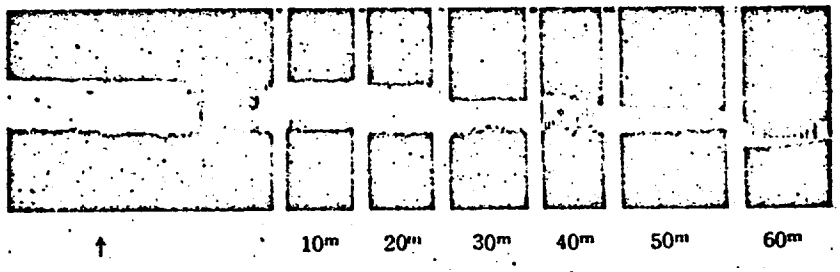
Sod. Cit. 0.0048%, 0.012mg/10g

Figure 2



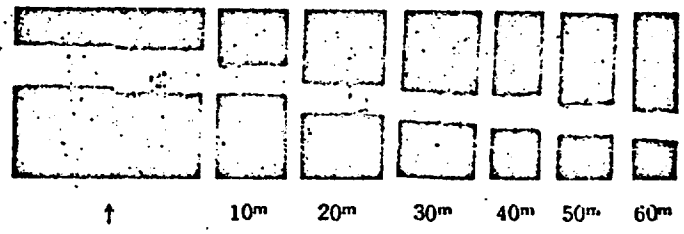
Sod. Ox. 0.0032%, 0.008mg/10g

Figure 3



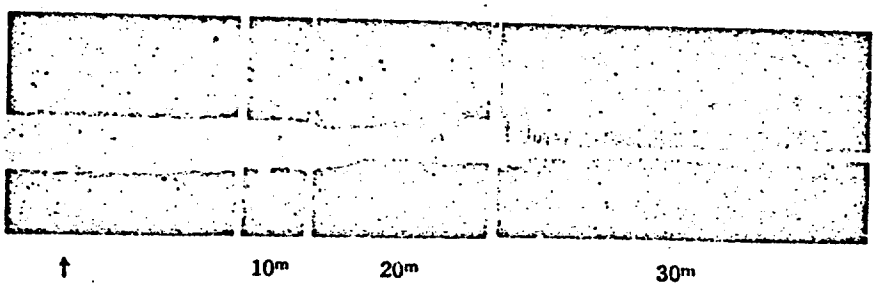
NaF 2%, 5mg/10g

Figure 4



Sod. Cit. 4.8%. 12mg/10g

Figure 5



Sod. Ox. 3.2%, 8mg/10g

Figure 6

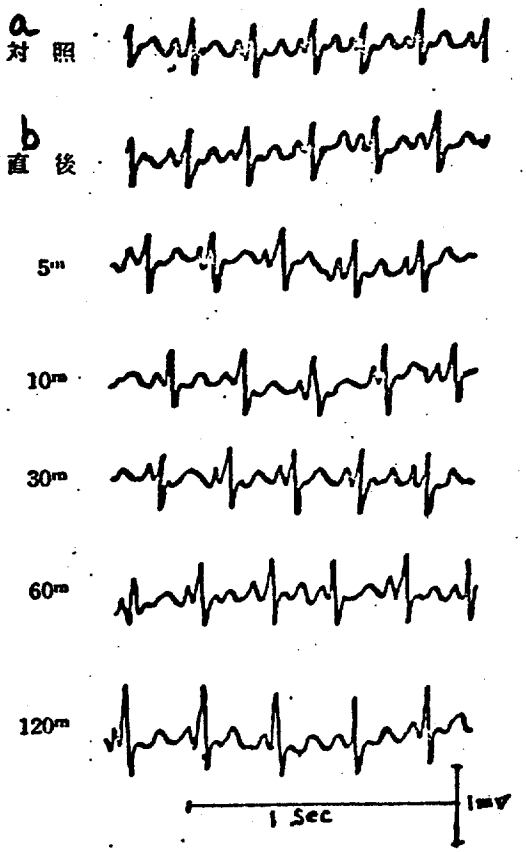


Fig. 7

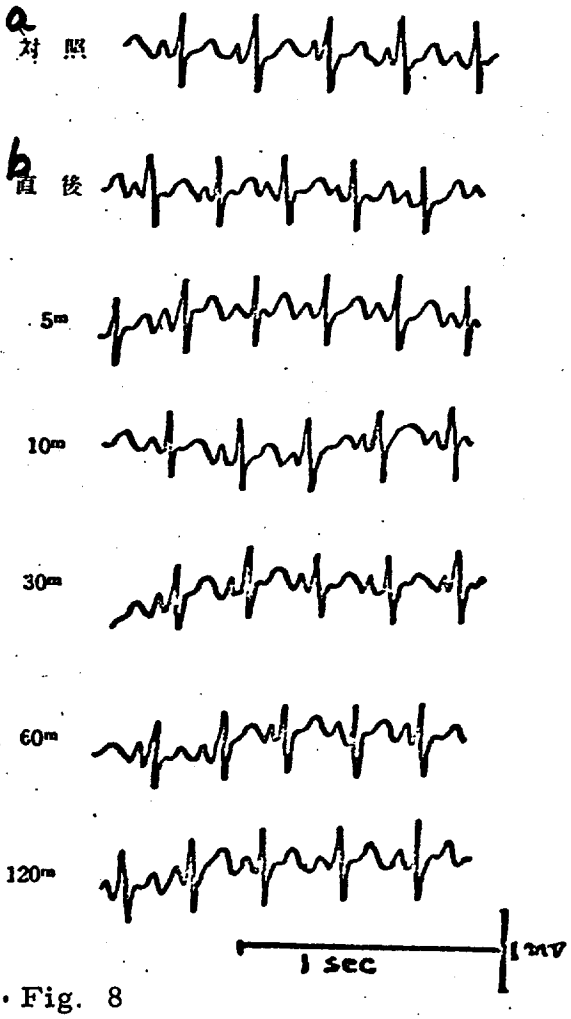


Fig. 8

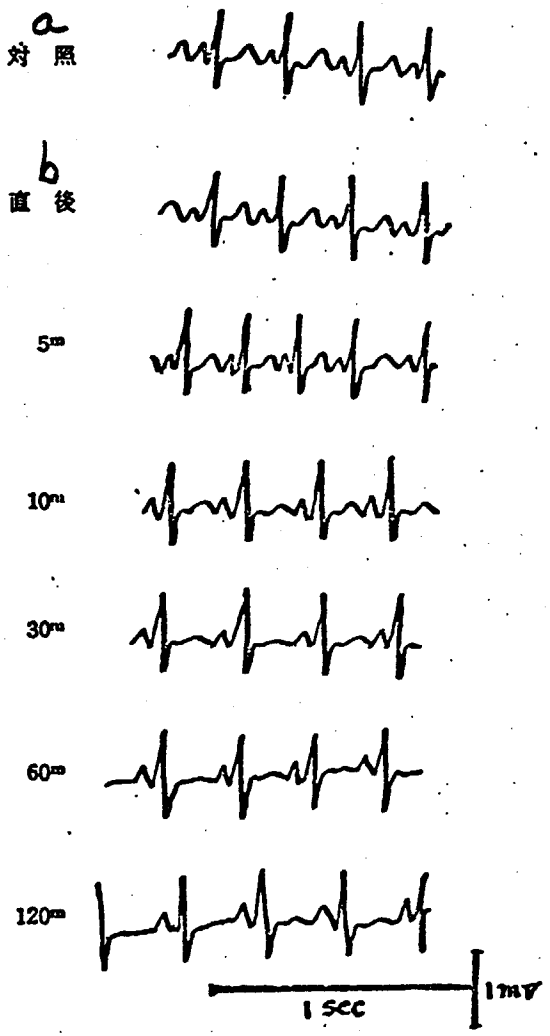

a
対照
b
直後
5m
10m
30m
60m
120m
1 sec
1mV

Fig. 9

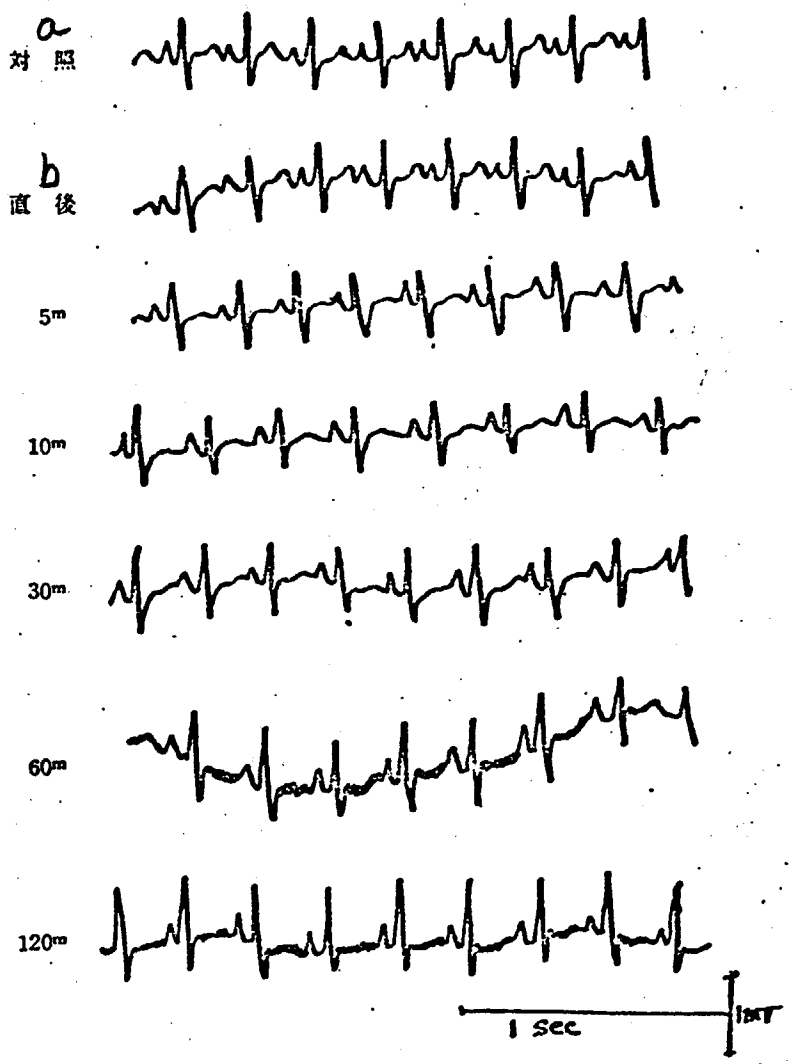

a
対照
b
直後
5m
10m
30m
60m
120m
1 sec

Fig. 10

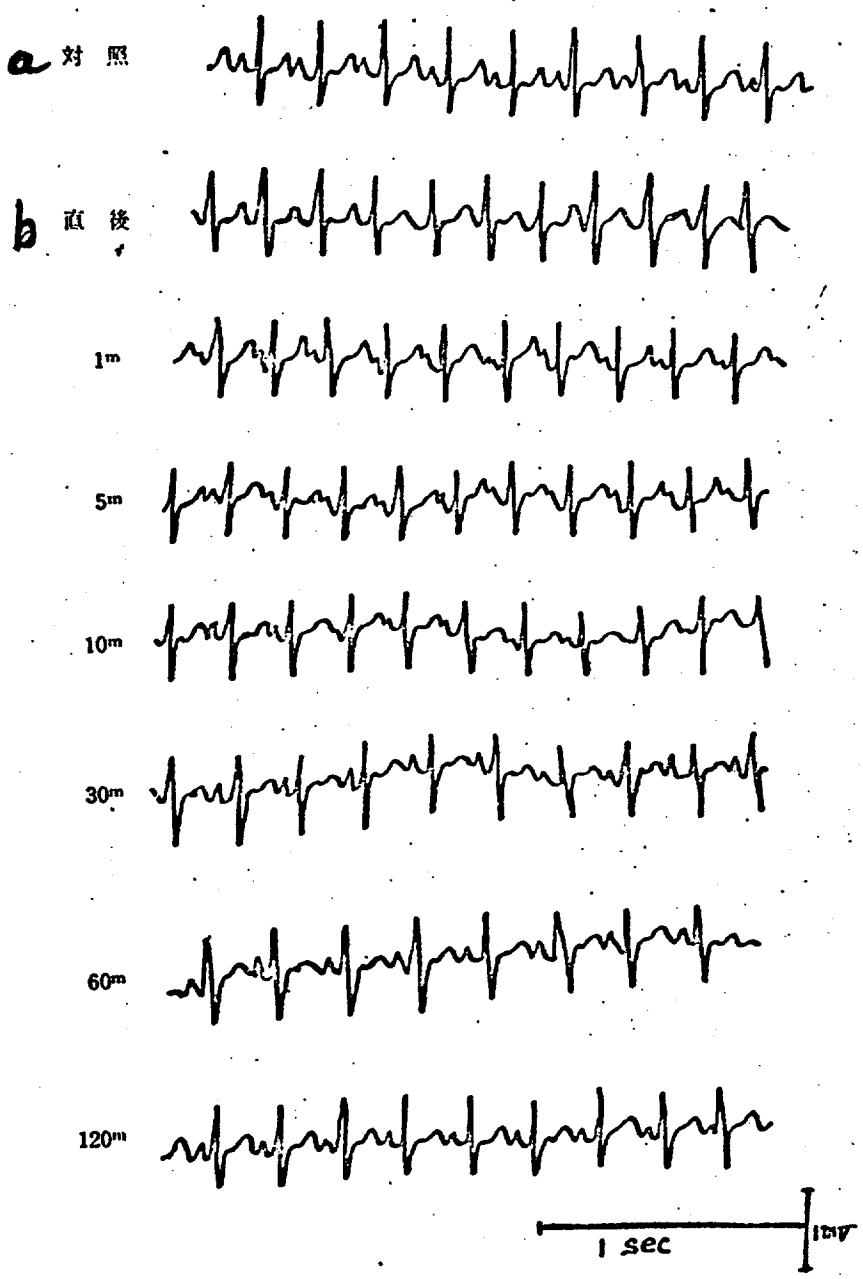


Fig. 11

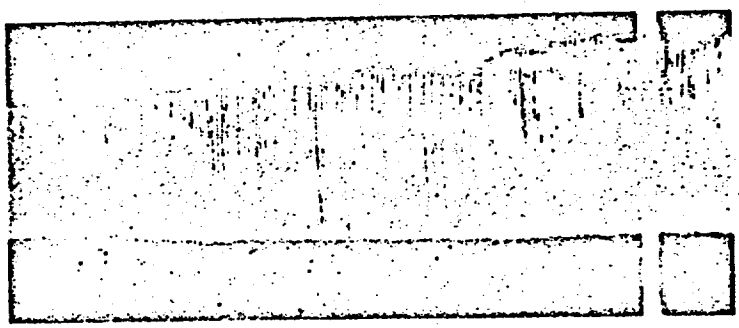
1.

NaF 0.001% R.

2.

Sod. Cit. 0.0048% R. R. R. R.

3.



Sod. Ox. 0.0032% R. R. 10m

Fig. 12

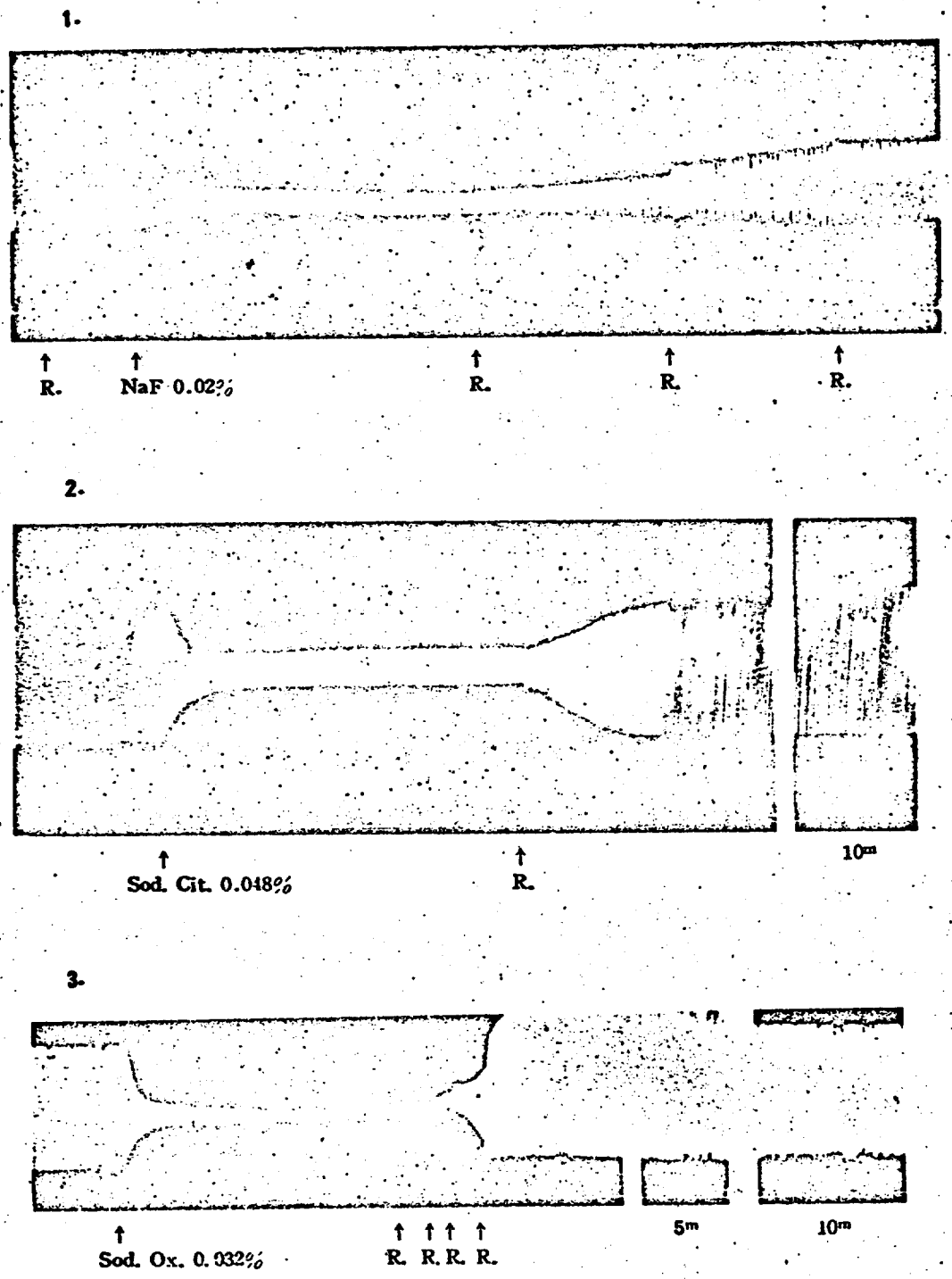
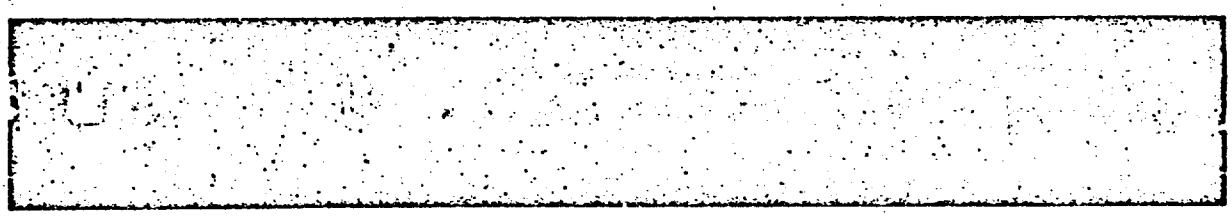


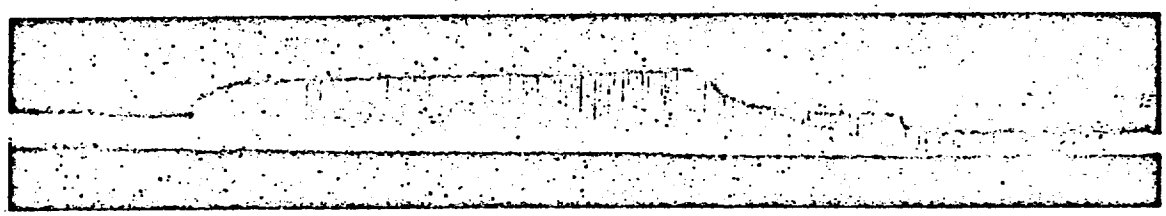
Fig. 13

1.



NaF 0.002% R. R. R. R.

2.



Sod. Cit. 0.0048% R. R.

3.

Sod. Ox. 0.0032% R. R. R. R. R.

Fig. 14

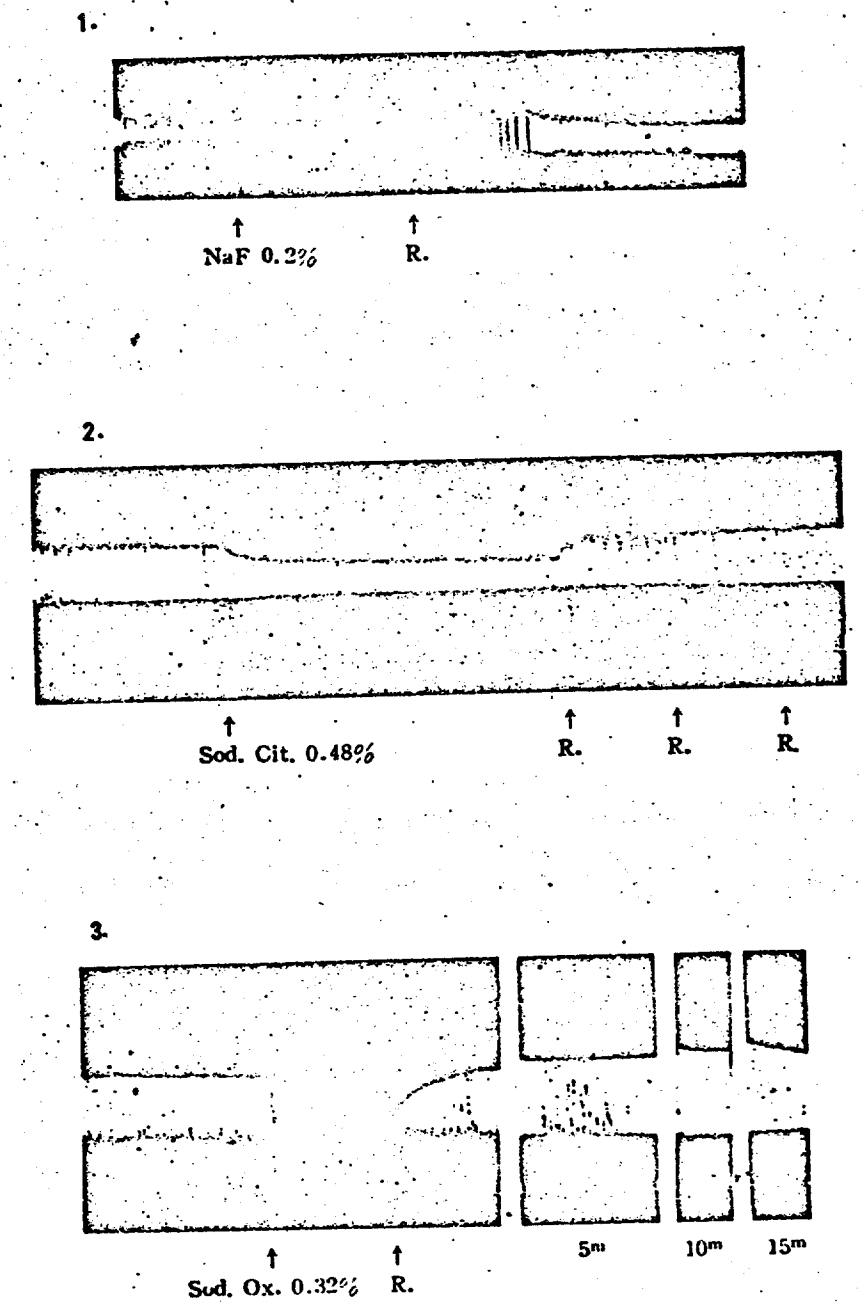
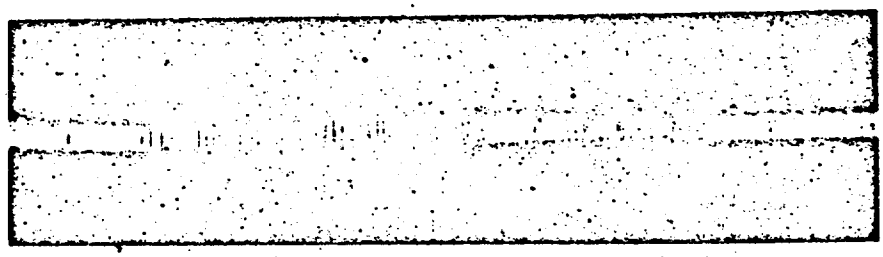


Fig. 15

1.



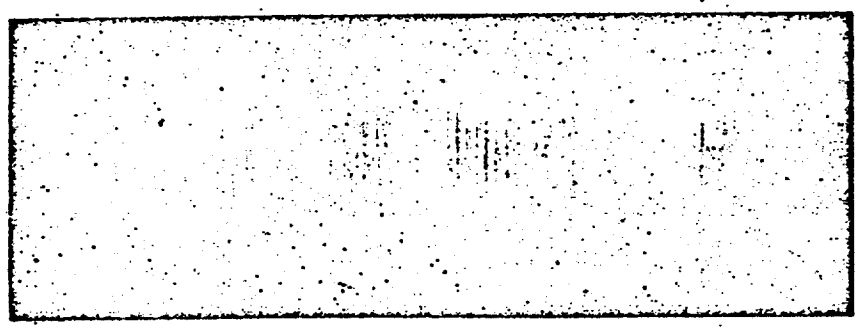
NaF 0.02% + $CaCl_2$ 80mg/dl

NaF 0.02% + $CaCl_2$ 160mg/dl

R.

R. R.

2.



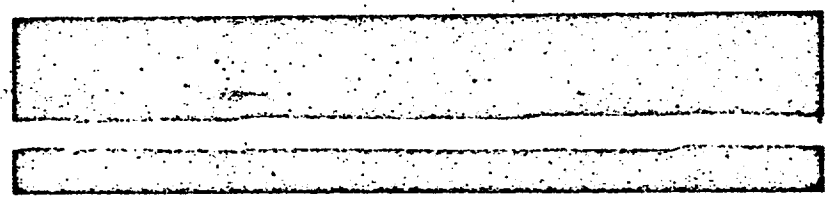
R.

Sod. Cit. 0.048% + $CaCl_2$ 80mg/dl

Sod. Cit. 0.048% + $CaCl_2$ 160mg/dl

R.

3.



Sod. Ox. 0.032% + $CaCl_2$ 80mg/dl

Sod. Ox. 0.032% + $CaCl_2$ 160mg/dl

R.

R.

Fig. 16

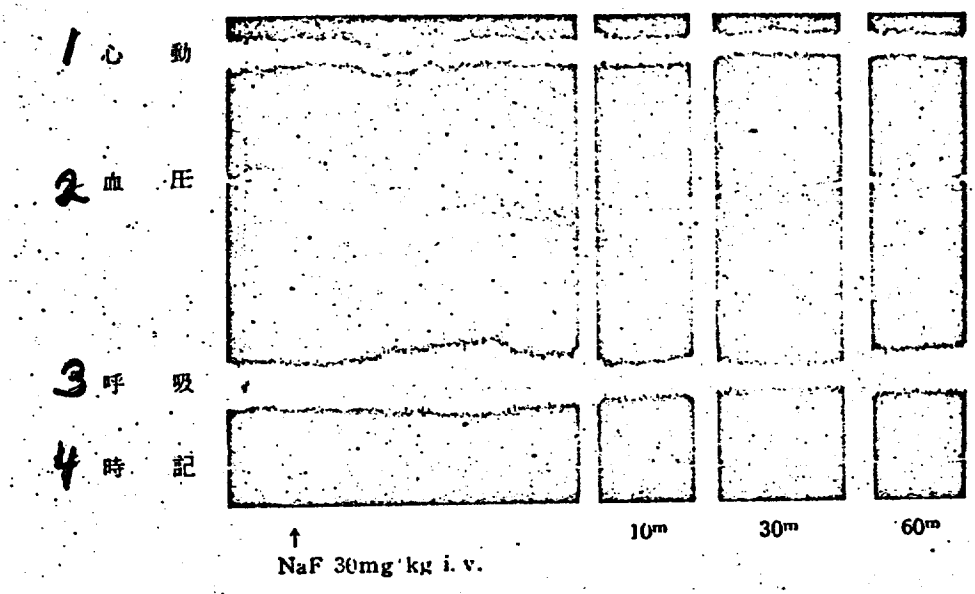

1 心 動
2 血 圧
3 呼 吸
4 時 記
10m
30m
60m
NaF 30mg/kg i. v.

Fig. 17

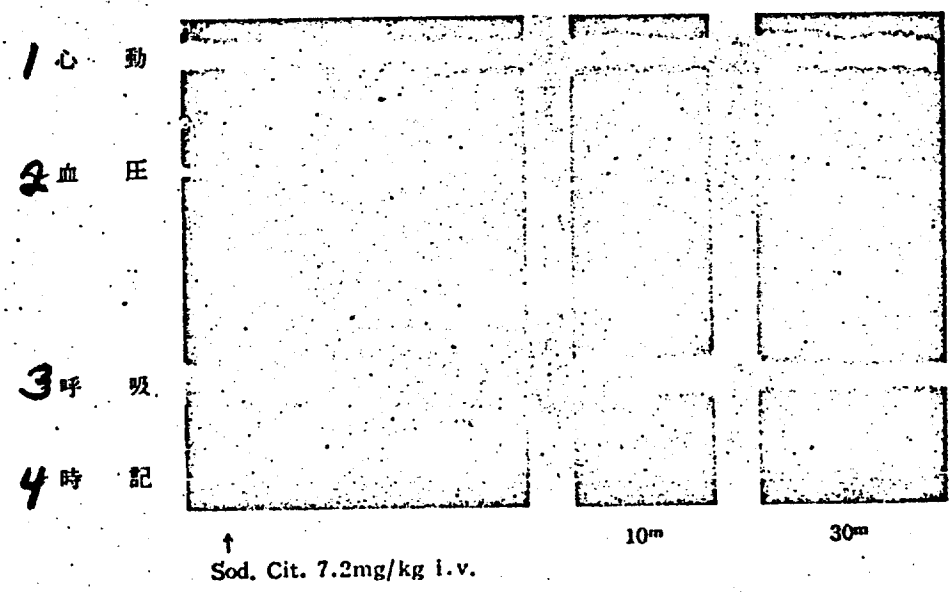

1 心 動
2 血 圧
3 呼 吸
4 時 記
10m
30m
Sod. Cit. 7.2mg/kg i.v.

Fig. 18

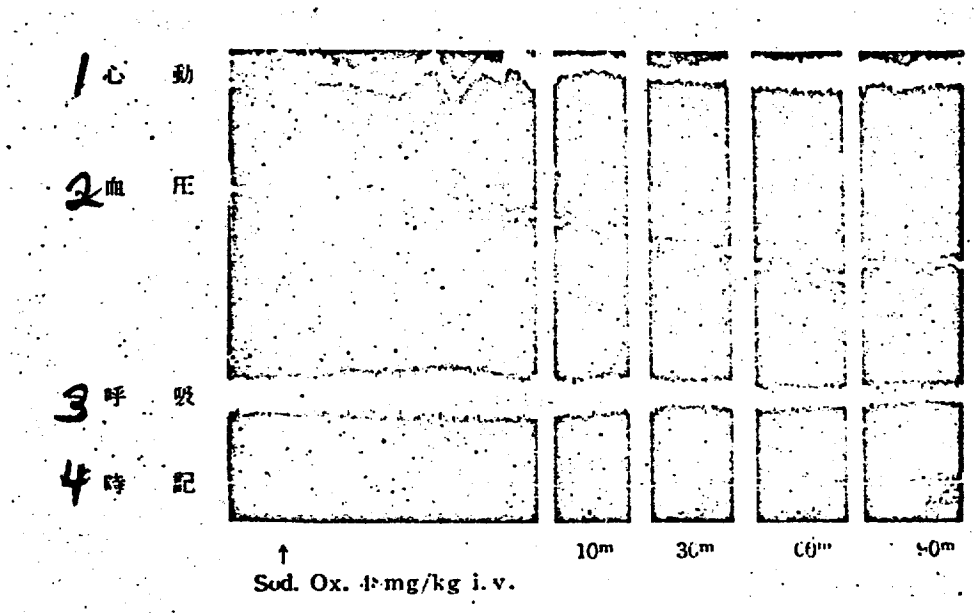

1 心 動
2 血 圧
3 呼 吸
4 時 記
10m
30m
60m
90m
Sod. Ox. 4mg/kg i.v.

Fig. 19

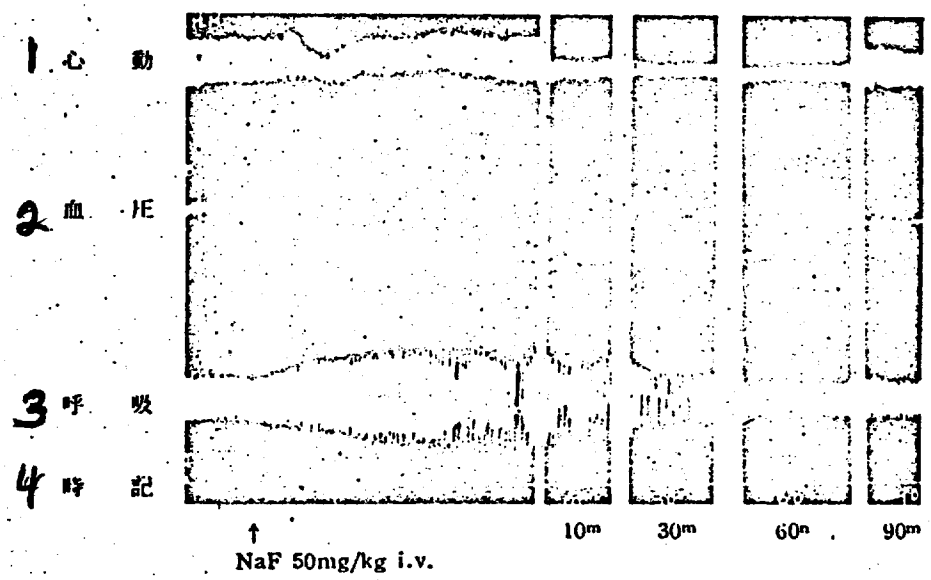

1 心 動
2 血 圧
3 呼 吸
4 時 記
10m
30m
60m
90m
NaF 50mg/kg i.v.

Fig. 20

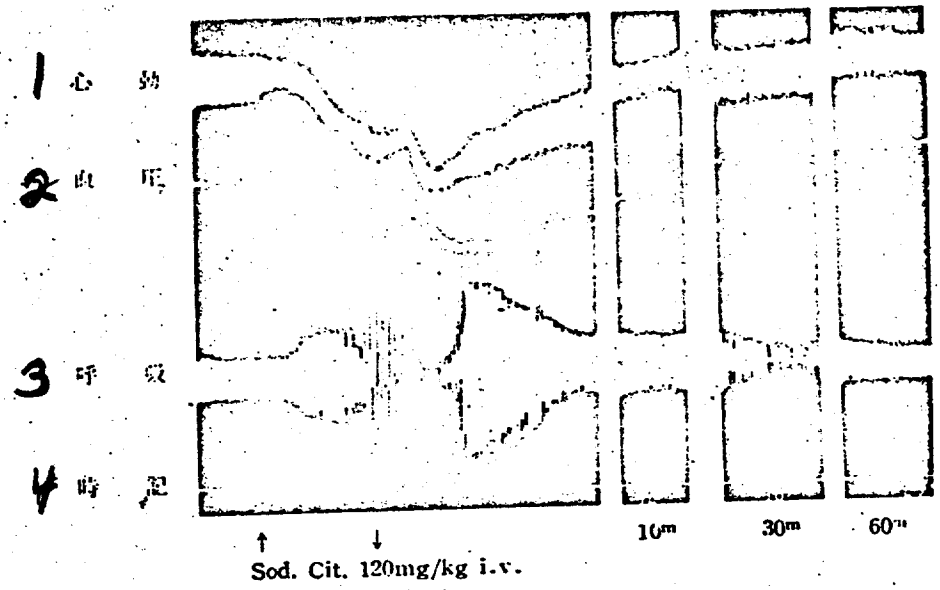


Fig. 21

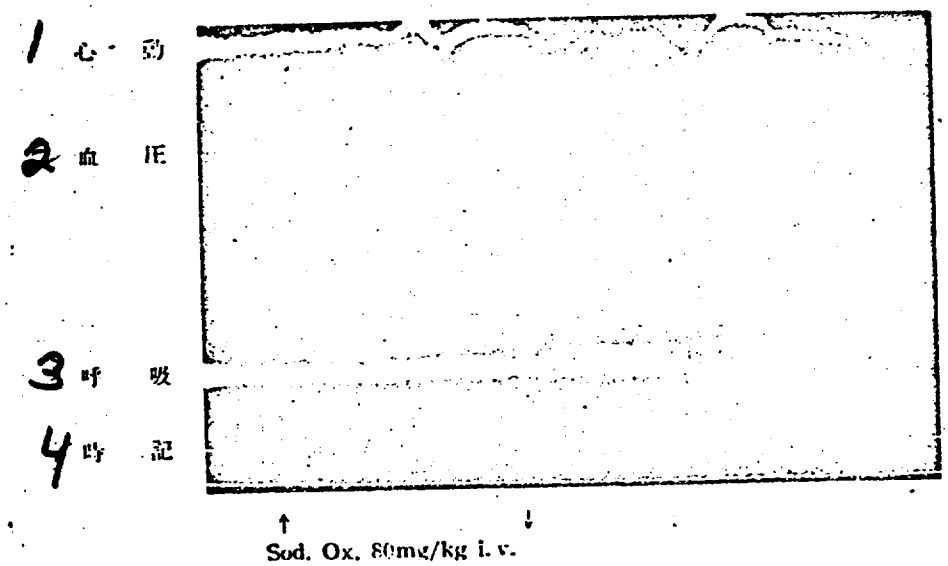


Fig. 22

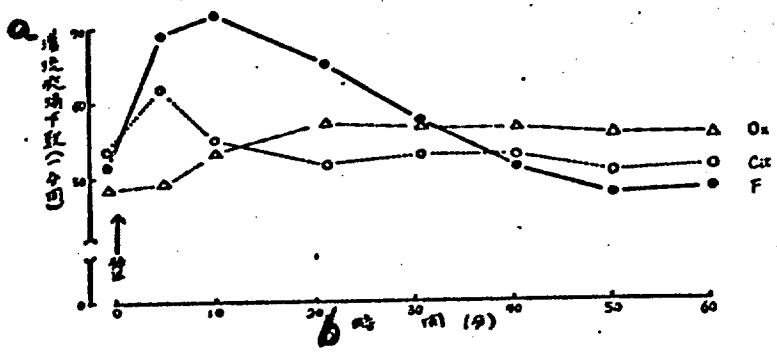


Fig. 23

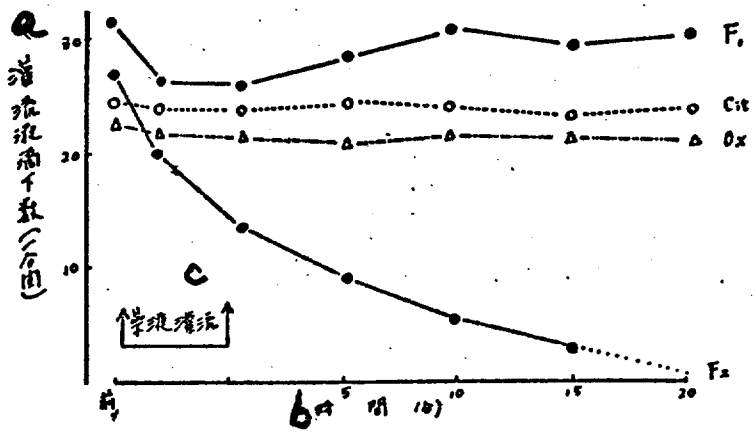


Fig. 24

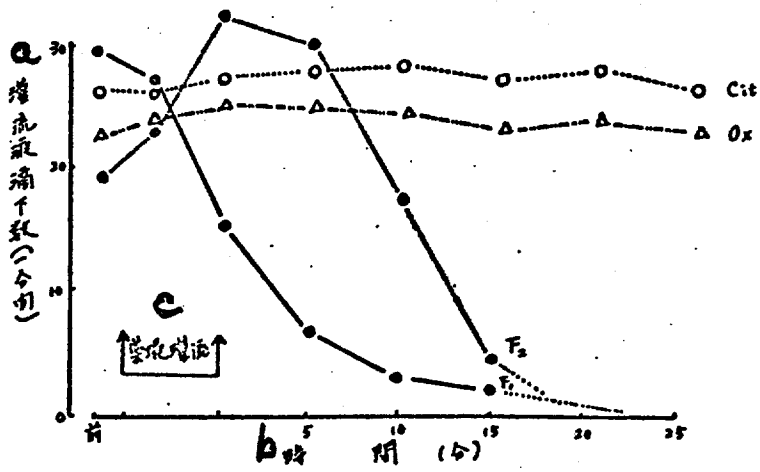


Fig. 25

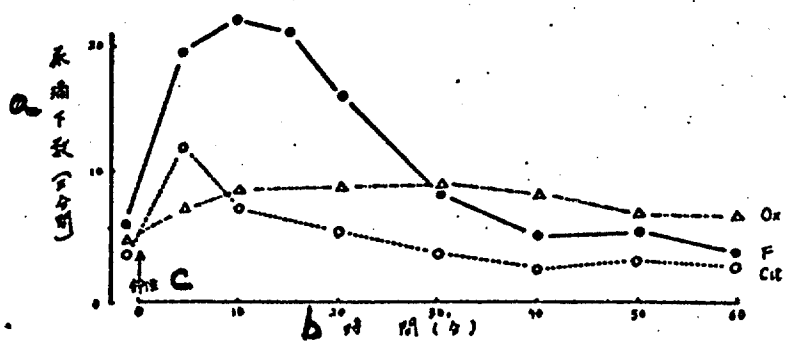


Fig. 26

↑ 10m 20m 30m

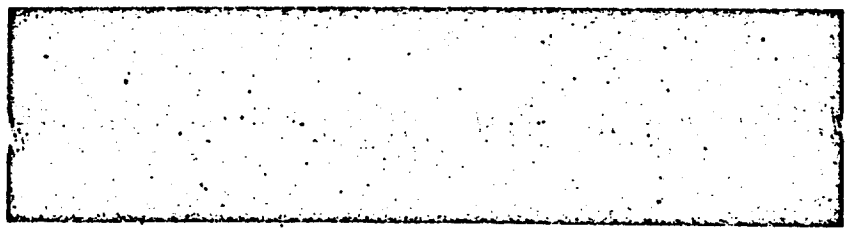
NaF 10mg/kg i.v.

Fig. 27

↑ 10m 20m 30m

Sod. Cit. 24mg/kg i.v.

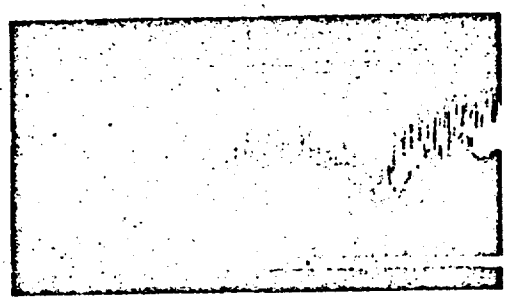
Fig. 28



↑ 10m 20m 30m

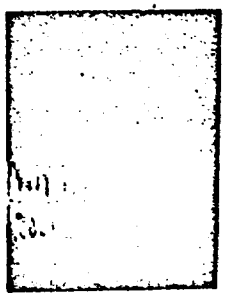
Sod. Ox. 16mg/kg i.v.

Fig. 29



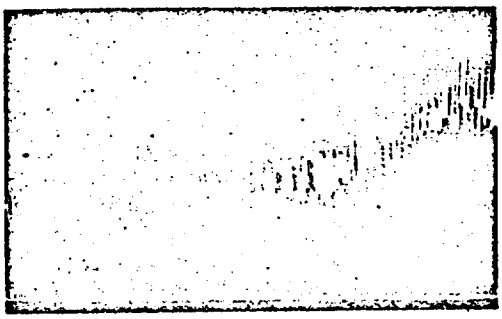
↑ NaF 10^{-5} ↑ Tyrode

1. 弗化ナトリウム



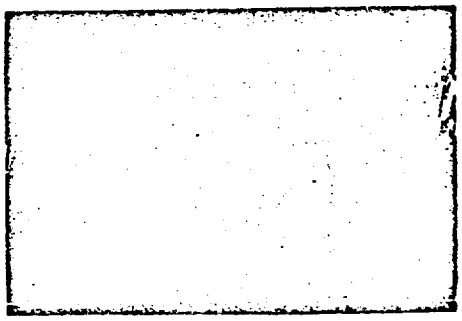
↑ NaF 10^{-3} ↑ Tyrode

2. 弗化ナトリウム



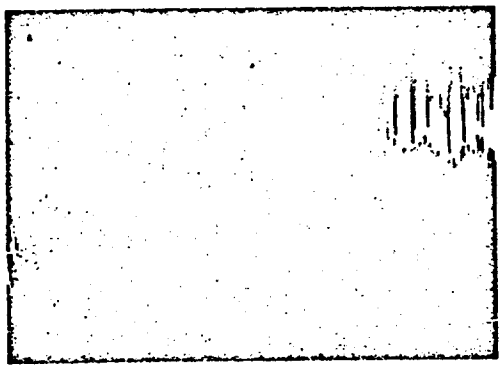
↑ Sod. Cit. 2.4×10^{-5} ↑ Tyrode

3. クエン酸ナトリウム



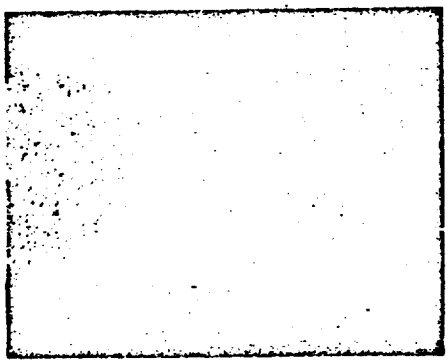
↑ Sod. Cit. 2.4×10^{-3} ↑ Tyrode

4. クエン酸ナトリウム



↑ Sod. Ox. 1.6×10^{-3} ↑ Tyrode

5. 蓚酸ナトリウム



↑ Sod. Ox. 1.6×10^{-3} ↑ Tyrode

6. 蓚酸ナトリウム

Figure 30

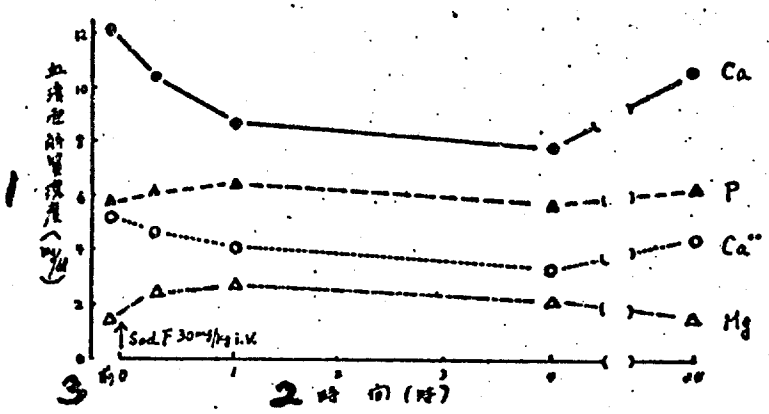


Fig. 31

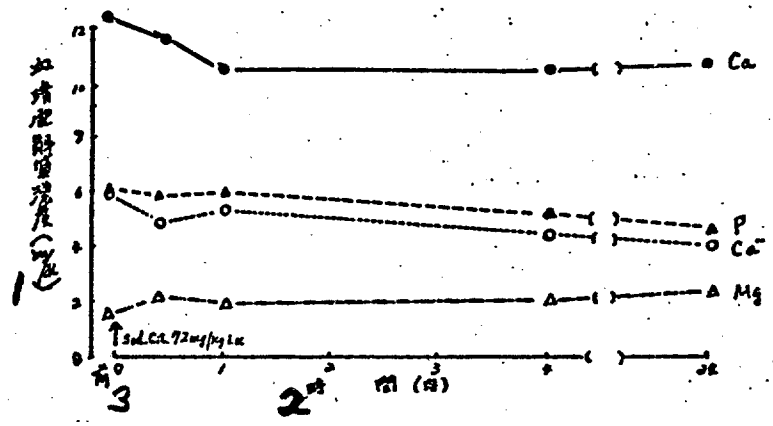


Fig. 32

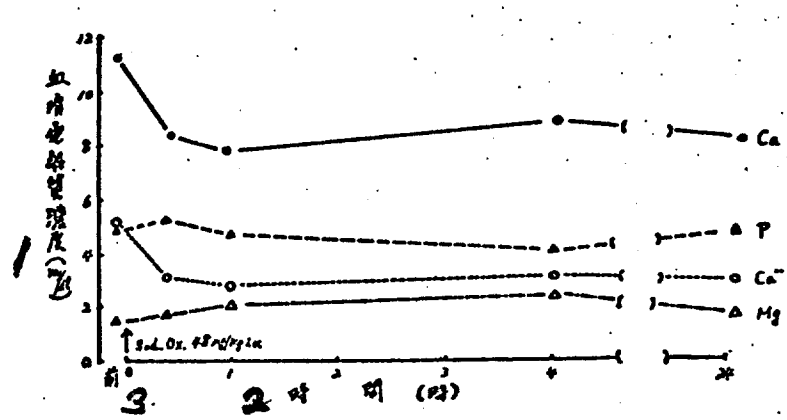


Fig. 33

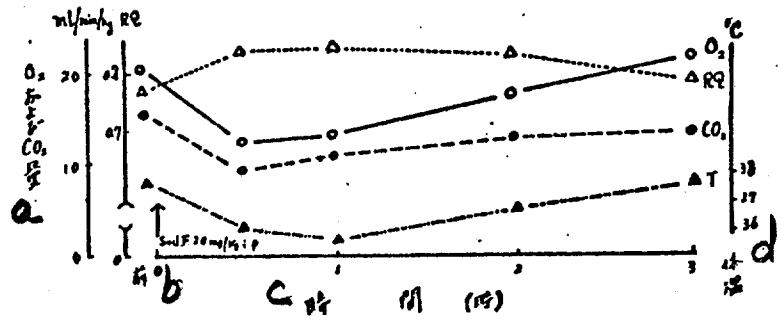


Fig. 34

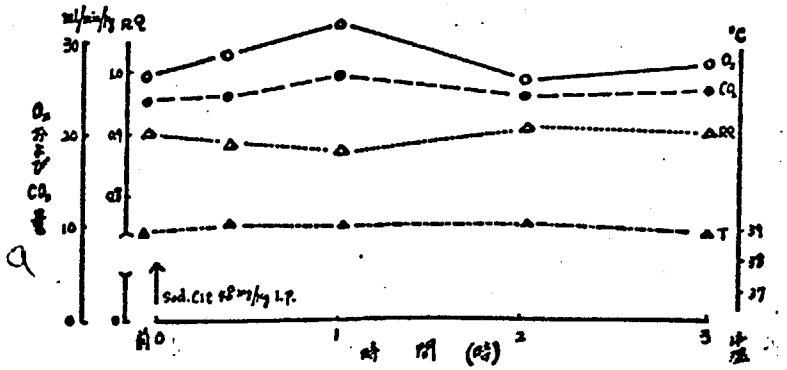


Fig. 35

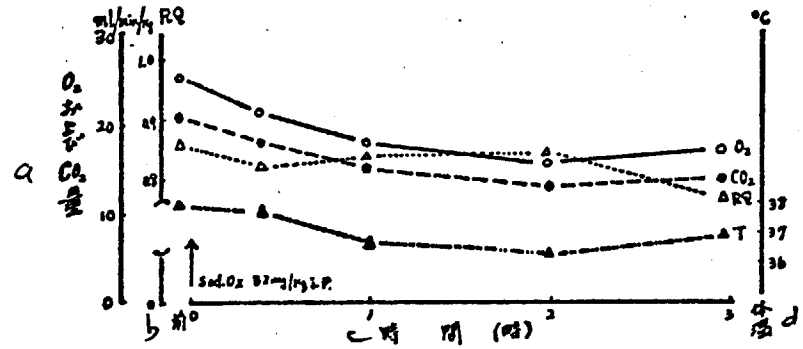


Fig. 36

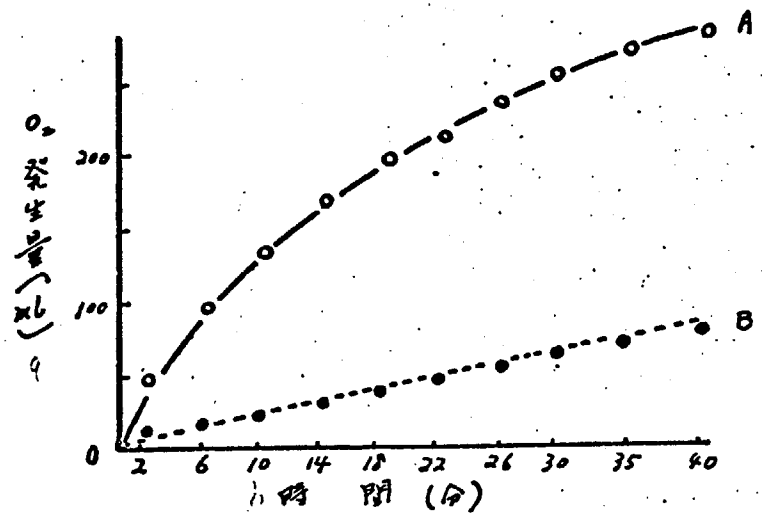


Fig. 37

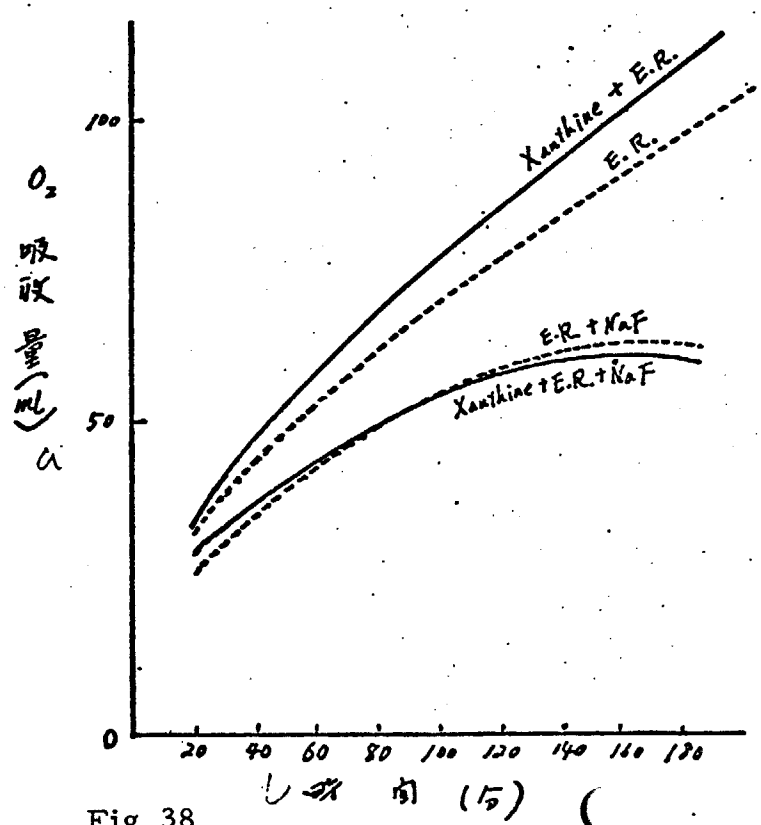


Fig. 38

1/3 ar FORL 6/6

Sodium Citrate 415

弗化ナトリウムの薬理学的研究とくにクエン酸ナトリウムおよび蓚酸ナトリウムとの比較

昭和35年4月30日受付

信州大学医学部薬理学教室（主任：赤羽治郎教授）
鈴木義政

Pharmacological Studies on Sodium Fluoride in Comparison with Sodium Citrate and Sodium Oxalate

Yoshimasa Suzuki
Department of Pharmacology, Faculty of Medicine, Shinshu University
(Director: Prof. J. Akabane)

緒言

弗素は Halogen 元素の一種で，自然界には地殻構成分として螢石 CaF_2 あるいは氷晶石 Na_3AlF_6 その他の形で広く分布している。さらに礦物界のみならず，動・植物組織中にも主として難溶性のカルシウム塩として存在している。

Heffter[1]および Sollmann[2]の各書の記述によると，Gay-Lussac & Berthold (1803～1805) がはじめてヒトの正常歯牙から弗素の存在を発見した。さらにその後の研究から，弗素は骨・歯牙に最も多く，ついで表皮・毛髪・爪・血液・乳汁その他の臓器組織中にも微量に存在することが明らかとなつた。

ここで弗素の生理的ないし薬理的作用に関する研究の歴史をみると，Rabuteau (1872)[3]がイヌおよびウサギに弗化ナトリウムを投与して，その中毒症状を観察したのが最も初期で，ついで Tappeiner[4][5]や Schultz[6]はヒキガエルで心運動抑制作用，運動神経末梢麻痺作用をみとめている。

その毒性に関しては Heffter, Goodman & Gilman[7]の各書の記載によると，多量を摂取すると激しい胃腸炎をおこすほか，諸種の酵素活動を阻害したり，低カルシウム血症や血液凝固抑制，血圧下降，心臓抑制，さらにてんかん様痙攣，甲状腺機能亢進のほか，肝・腎その他の臓器の退行性変化をまねくという。

Sollmann は原形質毒として局所粘膜の腐蝕あるいは壊死をきたすと述べ，Siegfried[8]は弗素は消化管粘膜上皮の組織呼吸を阻害するため必ずしも内用でなくても消化管粘膜の炎症が起るという。さらに Schultz[6]，Schwyzer[9]，Greenwood et al[10]，Mc Clure[11]，Gottdenker et al[12]，須川[13]らは大量を動物に注射すると血清カルシウムが減少することをみとめ，さいきん石見ら[14]もネコの血圧実験と平行して血清カルシウムの定量をおこない，急性弗素中毒症状は脱カルシウム Decalcification の結果であると論じている。

また近時，化学工業の進歩にともない氷晶石 Na_3AlF_6，弗化カルシウム CaF_2 や弗素を含む燐礦石などを取扱うアルミニウム製造，燐酸肥料製造，窯業などの工場において弗化物が粉塵や化学的処理によりガス状となつて，経気道的あるいは消化管から吸収されて工業中毒をおこすことがまれでないことが報告されている[15]。

これとは別に以前には食品の防腐剤として混入されたものによる中毒や，誤飲あるいは自殺の目的による中毒もしばしばみられた。美濃口[16]は数年前京都市内某小学校で，ウ歯予防の目的で弗化ナトリウム末内服投与のさい，誤つて過量を与えたため，集団的に急性弗素中毒が発生した例を報告している。

ヒトにたいする急性中毒量は Hodge & Smith[17]は 5～10g といい，美濃口は 6～8mg/kg と推定し，一定していない。致死量についても Goodman & Gilman は 5g 前後といい，美濃口は 8～18g と推定している。

上記のような急性中毒のほか，最近問題となつてきたのは，飲料水中に含まれる微量の弗素の長期にわたる摂取からおこる慢性中毒である。弗素の慢性中毒時の最も特徴ある変化の一つは斑状歯とよばれる歯牙の変化である。

斑状歯については，すでに1901年 von Eager[18]はナポリに近い火山地帯にこれが多いことを認め，これを Denti di Chiaie と命名し，ついでアメリカでも Black & Mc Kay (1910)[19]がアリゾナ州のコロラド川支流で同様の歯牙異常を発見し，斑状歯 Mottled enamel とよび，その原因を飲料水にもとめている。その後，水中微量弗素定量法の進歩によつて，Churchill[20]および Smith[21]ははじめて斑状歯の原因は飲料水の弗素によつて起ることを明らかにした。以来，各国においてこの方面の研究がおこなわれ，わがくににおいても正木・三村(1951)[22]，須川(1937)[23]によつて西日本各地の斑状歯地帯がはじめて発見され，その後多くの学者による研究が発表された。

このように斑状歯の原因が明らかとなるとともに，Dean ら(1941)[24]は斑状歯予防の目的で，飲料水からの弗素の減量をこころみたところ，児童の斑状歯の発生が減少したのみならず，ウ歯の発生もいちぢるしく抑制されることを発見した。すなわち幼少年の歯牙形成期に過量の弗素を含んだ飲用水を連用すると斑状歯が発生するが，微量の弗素を連用するとウ歯発生を予防することが明らかとなつた。以来，各国の研究者によつて弗化物のウ歯発生抑制効果について広汎な研究がおこなわれ，きわめて優れた成績がえられることが報告されている。

U. S. Public Health Service の発表[25]では，上水道に 1.0p.p.m. の濃度に弗化ナトリウムを混入すると，5年後に50～66%のウ歯減少率をみたといい，現在アメリカではウ歯予防の目的で 726 地区に上水道の弗素化がおこなわれ，1億5000万の人口が弗素化飲料水の供給をうけているといわれる。[7] わがくににおいても1952年以来，京都市山科地区の上水道に 0.6p.p.m. の弗素化が実施され，5年後の中間成績によれば，6～7才の学童の6才臼歯が，対照のそれぞれ65%，66%と著明なウ歯発生率の減少を示している。[16]

弗素によるウ歯発生予防法は上述の上水道の弗素化のほか，食塩[26]あるいは牛乳[27]中に混入し，または錠剤チューインガム[28]などの形で内用する方法や，弗化物の歯牙表面への塗布[29][30]などがおこなわれて，いずれも50～75%という優れたウ歯予防率を示している。

弗素のウ歯予防機構については諸説がある。一般に，歯牙の生成過程に弗素が作用すると，カルシウムと複塩の形をつくつて歯牙表面に沈着するが，Pincus (1949)，Volkor (1950) はこのものが菌感染路の閉塞の役割をするとかんがえており，Philips および Swartz (1948～1950) は歯牙硬度の増加によるといい，Cox (1952)[29]，Bibby & Kesteren (1940)，野口らは弗素の酵素抑制作用による醸酸機転の阻害によると主張し，その他殺菌説等もあり，なお意見の一致をみない。[31]

ヒトにおける慢性弗素中毒の所見は，前記斑状歯のほか，平田[32]は骨変化，および貧血を，高森[33]は高濃度弗素地域 (1.9～4.8p.p.m.) の調査から，小児・成人の心筋障害をみとめ，さらにウサギの慢性中毒実験でも同様の心筋障害がおこることを確認した。このように弗素中毒による生体の傷害はかなりはげしいものがあり，弗化ナトリウムは毒物として取り扱われている。

さらに弗素の薬理作用のうち特異なものとしては心臓にたいする作用と酵素抑制作用とがある。Vernon[34][35]，De Nitto[36]，Chang[37]らは心抑制作用を，Gottdenker ら[38]，Ferrannini[39]，Webb[40]らは哺乳動物の摘出心房条片について，はじめ一過性振幅縮小のち増大を，荻生[41]，瀬川[42]もウサギの心房条片で同様の所見をみ，さらに ECG 所見でも著明な抑制作用をみとめた。さらに大櫛ら[43]も弗化ナトリウム経口投与によるウサギ ECG の変化をみとめ，いずれも抑制効果について論じている。これにたいし Loewi[44]は低濃度弗素は Positive inotropic action を有することをみとめ，これを心筋のカルシウムとの反応によるものとし，Bennet ら[45]は銅イオンとの反応によるものと考え，さらに Covin & Berman[46]は酵素抑制作用によるものと反論している。

一方弗素の酵素抑制作用に関しては，Artus u. Huber[47]が最初に報告して以来，その急性中毒症状は酵素阻害と密接な関係を有するとかんがえている学者はかなり多い。Lippmann[48]は yeast 中の Anaerobic glycolysis は弗素により影響をうけ，また Phosphatase 中の Esterase も抑制され，その作用は Monoiodoacetic acid による抑制作用と類似していると報告している。Moruzzi[49]はネコの大動脈に弗化ナトリウムを注射して，Cortical glycolysis を瞬間的に抑制して大脳皮質の Electric activity を低下させたとのべ，McClure は Lipase，Esterase および Urease は強く抑制されるが，Proteolytic and diastatic enzyme はきわめて感受性が低く，唾液 Amylase はたとえ高濃度でも抑制されず，Alcohol 醸酵は抑制されたと報告している。また Sollmann は弗素の血液凝固抑制作用はカルシウムの添加によつても消失せず，Thrombine を添加したときはじめて凝固作用があらわれることから，弗素が Fibrin 形成を抑制するためであろうと考えて，酵素阻害作用を重要視している。また藤本[50]，石見ら[51]は Flavin 酵素系の阻害について，米沢[52]，帆足[53][54]はウ歯細菌の酸醸酵阻害をみとめるなど酵素系にたいする弗素の作用についての報告はすくなくない。

以上のごとく弗素に関する基礎的研究はすくなくないが，その毒性ならびに一般薬理作用について系統的に研究されたことは少なく，またその作用機序に関しても意見の一致をみない点が多い。すなわち弗化物の生体にたいする毒性および薬理作用が，たんに生体

内のカルシウムと結合して，生体内活性カルシウム（Ca^{++}）量を減少させる（脱カルシウム作用）ことにのみ由来するのか，あるいは弗素イオンじしんの特異作用にも関連してはいないかとの疑問がある。

著者はウ歯予防薬として最も広く応用されている弗化ナトリウムをえらび，その毒性ならびに一般薬理作用を研究するとともに，弗素の生体内における作用機序を明らかにする目的で，弗素と同様にカルシウムと結合する性質を有する薬物のうち，クエン酸ナトリウムおよび蓚酸ナトリウムをえらび，これらと弗化ナトリウムの薬理作用と比較しながら実験をすすめた。すなわちこれら3種の薬物について，in vitro で同一量のカルシウムを結合すると考えられる計算値の等価濃度薬液（同一 Equivalent concentration）をつくり，これら薬物の in vivo の作用について比較検討をこころみた。さらにこのさいの血清カルシウム，マグネシウムならびに無機燐の変動をもしらべた。また弗化ナトリウムの血液 Catalase，肝 Xanthine oxidase にたいする作用，および呼吸ガス代謝にたいする作用についても実験をおこなつた。

実験方法ならびに実験成績

実験薬物の調製：弗化ナトリウム NaF，クエン酸ナトリウム $C_6H_5Na_3O_7 \cdot 2H_2O$ および蓚酸ナトリウム $Na_2C_2O_4$ は，いずれも関東化学製の純品をもちい，生理食塩水溶液または Ringer 溶液とし，各薬物はすべて同一量のカルシウムと対応する等価濃度（同一 Equivalent concentration）となるよう調製して実験に供した。

すなわち F が Ca と結合して CaF_2 となるためには，$CaCl_2$ 1分子にたいして NaF の2分子を要し，$C_6H_5Na_3O_7 \cdot 2H_2O$ が $Ca_3(C_6H_5O_7)_2 \cdot 4H_2O$ となるには $CaCl_2$ 3分子にたいしてその2分子を要し，さらに $Na_2C_2O_4H_2$ の場合は $CaCl_2$ 1分子にたいしてその1分子を必要とする。これを同一 Equivalent concentration で比較概算すると，NaF:$C_6H_5Na_3O_7 \cdot 2H_2O$: $Na_2C_2O_4$ = 1 : 2.4 : 1.6 の濃度比において等価となる。

これら3種薬物の薬理作用が，生体内における脱カルシウムによつて説明しうるものとすれば，同一 Equivalent concentration において，3者は同等の作用が現れてよいはずである。著者の実験はすべてこの比率のもとに各薬物の等価濃度溶液を調製し検討した。なお使用薬物の pH はそれぞれ，1%弗化ナトリウム 7.5，2.4%クエン酸ナトリウム8.2および1.6%蓚酸ナトリウム7.3（いずれも生理食塩水溶液）であつた。

各研究項目の実験方法はそれぞれの項目において記述した。

I 毒性ならびに急性中毒症状

実験方法

体重 20g 前後の純血性成熟マウスを，混合飼料で飼育し実験に供した。各薬物はいずれも生理食塩水に溶解し，注射全量が各薬物あたり 0.5ml をこえないように種々の濃度に薬液を調製し，これを腹腔内に注射した。なお急性中毒症状の観察は薬液注射後 24^h にわたりおこない，LD_{50} の算出は Up and down method によつた。実験時の室温は 20～23°C であつた。

実験成績

a. 弗化ナトリウム：100mg/kg では注射後まもなく呼吸は促迫かつ不整となり，全身の立毛をきたし，5^m 前後にいたると流涎，流涙がみられる。約 10^m 後には外来刺激にたいする反応は活潑となり，自発運動は亢進し，不穏状ないしは興奮状をしめすが，20^m 前後となると，かえつて刺激にたいする反応性は低下してくるが，30^m 後には軽度の線維性搐搦や間代性痙攣があらわれ，これらはしだいにはげしくなり，ときには痙攣状の跳躍がみられることもある。ついで後肢麻痺があらわれて歩行は不能となり腹位をとるにいたるが，40^m ころには四肢軀幹を伸展し，ついには横転して激しい間代性痙攣をつづけるとともに鼻尖にチアノーゼがあらわれ，またしばしば排尿，下痢がみられる。このような状態のもとに，多くの例は 1^h 前後に呼吸麻痺をきたして死亡した。70mg/kg でもほぼ上記と同様の激しい中毒症状をきたし，1^h 前後で死亡する例が多いが，1^h 以上を経過した例では生存することが多く，徐々に中毒症状は回復にむかう。LD_{50} は 71.5～75.8mg/kg（危険率：5%）。

b. クエン酸ナトリウム：弗化ナトリウム100mg/kg に相当する 240mg/kg ていどの注射では，外見上まつたく正常と異るところなく，直後わずかに呼吸数が増加する傾向がみられるにすぎない。2.4g/kg では注射直後から呼吸が著明に促迫かつ不整となり，陰茎は腫大状を呈し，動物は不穏，興奮状態となり，さかんに走り廻る。10～15^m 後には全身の立毛や尾の弓状またわ上方への挙上がみられ，やがて四肢痙攣，牙関緊急，強直性痙攣があらわれる。しかし多くの例が30～40^m 後には数回の痙攣にたえてしだいに平常にもどり，2～3^h 前後で回復する。3.6g/kg 前後の注射ではこれらの興奮症状はさらに強く，かつ早く現われ，10^m 前後で後肢麻痺，横転，反弓伸張，四肢痙攣，強直性痙攣についで呼吸麻痺におちいつて20～30^m 後に死亡する例が多い。LD_{50} は 2.67～3.09g/kg（危険率：5%）。

c. 蓚酸ナトリウム：弗化ナトリウム 100mg/kg に相当する 160mg/kg ていどでは著明な中毒症状はみられず，呼吸不整，不穏状がみられるが，3～4^h で多くの例が回復した。800mg/kg では注射直後より呼吸は促迫状を呈し，不整となり，不穏状を呈するが，まもなく全身の立毛がみられ，やがて背をまるめてうずくまり，外来刺激にたいしても反応性は低下し，自発運動も減弱して歩行しなくなる。10^m ころには痙攣状に跳躍したり，横転して激しい間代性あるいは強直性痙攣をくりかえす。15^m ごろにいたると眼球は突出し，四肢を伸展させ線維性搐搦をきたし，ついで呼吸麻痺におちいつて死亡する。LD_{50} は 345～385mg/kg（危険率：5%）。

II 心臓にたいする作用

A. 生体内心臓にたいする作用

1. カエルの生体内心臓にたいする作用

実験材料および方法

体重 20～30g のトノサマガエルを使用し，10% Urethane 50mg/10g 注射により麻酔し，呼吸運動の停止をまつて，カエルを背位に固定し，心臓を露出し，Engelmann 法により心運動をヘーベルをもつて煙紙上に描記させた。薬液は各種濃度のものを Ringer 液に溶解して大腿リンパ嚢内に 0.5ml 以下となるように注射し，60^m 間観察した。

蓚酸ナトリウムは Ringer 溶液とすると白濁するため使用前によく振盪した。このさいの室温は 24～28°C。

実験成績

a. 弗化ナトリウム：2×10^{-6} 溶液，0.005mg/10g ではわずかに拍動数の減少傾向がみられるにすぎない。2×10^{-5}，0.005mg/10g では注射後 30^m ころよりわずかに振幅増大傾向を示すが，拍動数はかえつて減少傾向を示した。緊張には大部分に変化をみとめないが，小数例に低下を示したものもあつた（第1図）。2×10^{-5}，0.05mg/10g では注射後 30～40^m ころより振幅の増大がみられ，拍動数もわずかに増加した。このさいの緊張はやや低下傾向を示した。

0.2%，0.5mg/10g では7例中4例が注射後 5～10^m ころより，わずかに振幅減少と拍動数増加の傾向を示し，3例はこれと全く逆の振幅増大と拍動数の減少を示した。緊張は全例低下を示した。2%，5mg/10g s.c. では20～30^m ころから著明な振幅および拍動数の減少と緊張低下をみとめ，30～40^m 後にブロックがみられた（第4図）。

b. クエン酸ナトリウム：4.8×10^{-6} 以下の濃度では変化なく，4.8×10^{-5}，0.012mg/10g では注射後 40^m ころから軽度の振幅増大と拍動数減少がみられた（第2図）。4.8×10^{-4}，0.12mg/10g では振幅に著変なく，拍動数のみわずかに減少した。しかし 0.24%，0.6mg/10g，0.48%，1.2mg/10g でもこの程度の変化がみられるにすぎなかつた。4.8%，12mg/10g では振幅および拍動数は軽度の減少を示した（第5図）。緊張は 4.8×10^{-5}，0.012mg/10g 以上で全例低下を示し，濃度が高くなるほど著明であつた。

c. 蓚酸ナトリウム：3.2×10^{-6}，0.0008mg/10g s.c. 以下の濃度では変化なく，3.2×10^{-5}，0.008mg/10g s.c. では軽度の振幅増大と拍動数の減少がみられ，緊張には変化がなかつた（第3図）。3.2×10^{-4}，0.08mg/10g s.c. ではきわめて軽度の振幅減少と拍動数増加がみられた。これ以上の濃度，量では緊張はいずれも低下し，濃度，量が高くなるにつれてその傾向は著明にあらわれた。

0.32%，0.8mg/10g s.c. では緊張の低下はさらに強く 20^m 後に半数例にブロックを生じた。3.2%，8mg/10g では 15～20^m 後ころより全例に著明な振幅ならびに拍動数の減少をきたし，30^m 前後にはブロックがあらわれた（第6図）。

2. ウサギの ECG にたいする作用

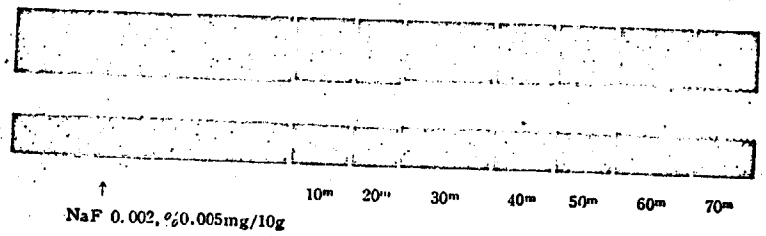
実験材料および方法

体重 2.0～2.5kg の雄性成熟ウサギを Nembutal 30mg/kg 腹腔内注射で麻酔してから，絶縁した固定台上に背位に固定した。1/4 注射針を誘導電極とし四肢の一定部位に刺入固定した。記録装置は福田エレクトロ社製の全交流撮影式心電型（FAC-2 型）を使用し，標準肢誘導の第2誘導を記録した。

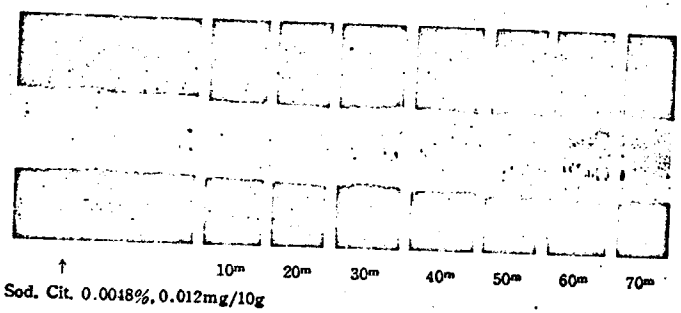
ウサギは実験前 12～16^h 絶食させ，室温は 18～20°C に保つように注意した。心拍状態が一定となるのをまつて記録をとり対照とした。ついで耳介静脈より各薬液（生理食塩水溶液として）を徐々に注射し，直後，5^m 後，10^m 後，30^m 後，1^h 及び 2^h 後を記録した。

実験成績

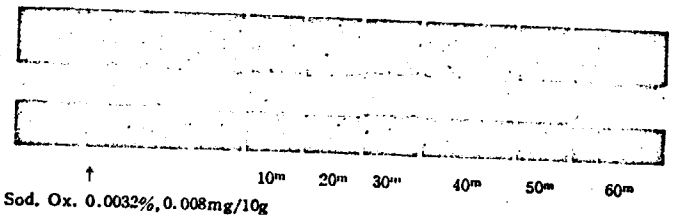
a. 弗化ナトリウム静注：5mg/kg 以下ではほとんど変化なく，30mg/kg では4例中3例に心拍数の減少を認め，30～60^m ころ一旦回復の傾向を示すものもあるが，再び減少して 2^h 値は前値より 50～60/min. 減少した。1例にはほとんど変化がみられなかつた。また 5^m～60^m ころ Q-T の軽度延長をみたものもあつた（第7図）。50mg/kg では4例中3 5^m ころ



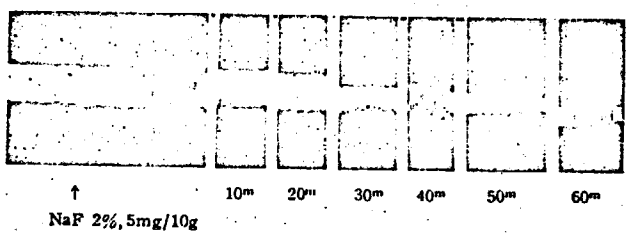
第 1 図　　生体内カエル心にたいする弗化ナトリウムの作用



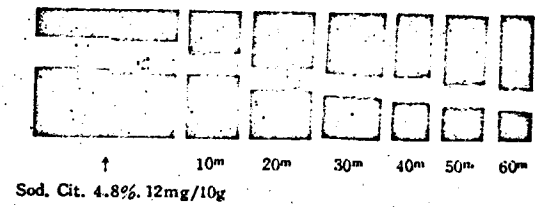
第 2 図　　生体内カエル心にたいするクエン酸ナトリウムの作用



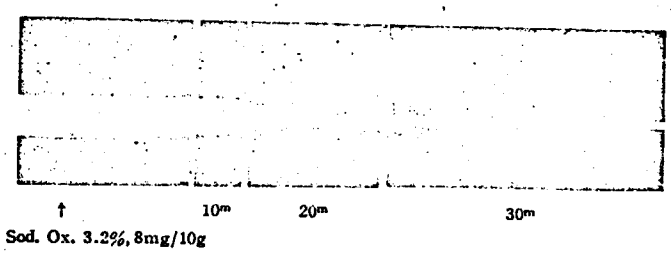
第 3 図　　生体内カエル心にたいする蓚酸ナトリウムの作用



第 4 図　　生体内カエル心にたいする弗化ナトリウムの作用



第 5 図　　生体内カエル心にたいするクエン酸ナトリウムの作用



第 6 図　　生体内カエル心にたいする蓚酸ナトリウムの作用

からS-Tの下降がみられ，時間とともに著明となり，2^h後には全く平低化したが，逆転をみた例はなかつた。また10^mころからQ-Tの延長と心搏数の減少がみられ，2^h後もなお回復はみられなかつた。しかし1例には直後から10^mまで心搏数が軽度増加をみたが30^mで前値にもどり，以後は他の例と同様の変化を示した（第10図）。

b. クエン酸ナトリウム静注：72mg/kgでは，直後から心搏数の増加がみられ60^mまで続くが，以後回復を示し2^hには全く前値にもどつた。波型にはいずれもほとんど変化をみなかつた（第8図）。これ以下の量ではほとんど変化はみられなかつた。120mg/kgでは，直後から10^mころまで心搏数は30～40/min. 増加を示すが30^mころには回復し，以後かえつて減少を示し，1^hで最も著明（40～50 min. 減少）となるが，2^hにはほとんど回復した。波型には直後から5^mまで全例にP-Q間隔の短縮がみられ，P波はほとんどみとめがたくなることが多いが，30^mにはほぼ回復し，同時にQ-T延長をみるが，いずれも2^hには回復した（第11図）。

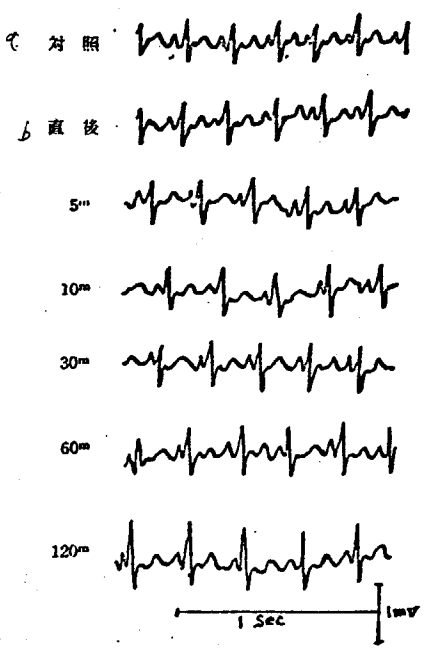
c. 蓚酸ナトリウム静注：48mg/kgでは，5^mころから不整脈が現れ，心搏数は軽度増加を示すが，30^mにはかえつて減少し，Q-T延長とS-T下降がみられ，時間とともにさらに著明となる（第9図）。72mg/kgでは多くの例が注射後から呼吸興奮，流涎，流涙を伴い，暴れ牙関緊急をきたし，筋電流が多く，10^mころには瞳孔散大をきたして呼吸停止し，ほとんどECG測定は不能であつた。

B. 摘出心臓にたいする作用

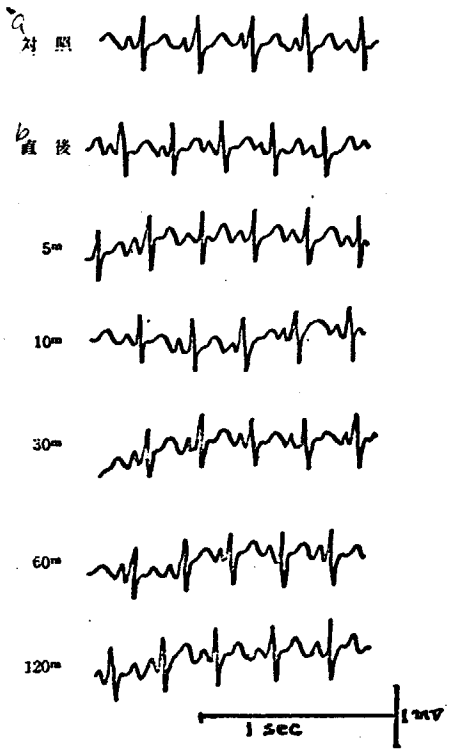
1. ヒキガエル摘出心臓にたいする作用

実験材料及び実験方法

体重300～500gのヒキガエルを使用し，無麻酔で，背位に固定し開胸して，心臓を露出，八木式の通法にしたがつて心臓を摘出し，Ringer液で灌流した。静脈圧は$2cmH_2O$とし，心室の状態が一定してから心室にセルフインをかけ，ヘーベルをもつて心運動を煤煙紙上に描記させた。薬液は各濃度のRinger溶液として灌流し，薬液の作用を解察したのちには再びRinger液と交換してその作用を観察した。



第7図　弗化ナトリウムのウサギECGにたいする作用
NaF 30mg/kg i.v.



第8図　クエン酸ナトリウムのウサギECGにたいする作用
Sod. Cit. 72mg/kg i.v.

実験成績

a. 弗化ナトリウム：10^{-8}溶液の灌流ではほとんど変化なく，10^{-7}溶液では5例中1例に極めて軽度の振幅減少をみたが，Ringer液交換とともに前値にもどつた。10^{-6}溶液では5例中3例に軽度の一過性振幅減少のち増大傾向をみとめ，2例は灌流中には変化なく，Ringer液交換ごに軽度の振幅増大をみ，1例は軽度の振幅増大のまま一定し，Ringer液交換とともに旧に復した。これまでの濃度では，緊張及び搏動数にはほとんど変化がみられなかつた。10^{-5}，2×10^{-5}溶液では3例中1例に著変なく，2例に薬液灌流直後より軽度の振幅減少と搏動数の増加がみられ，緊張はやや低下の傾向を示したが，いずれもRinger液交換により漸次回復した（第12図，1）。

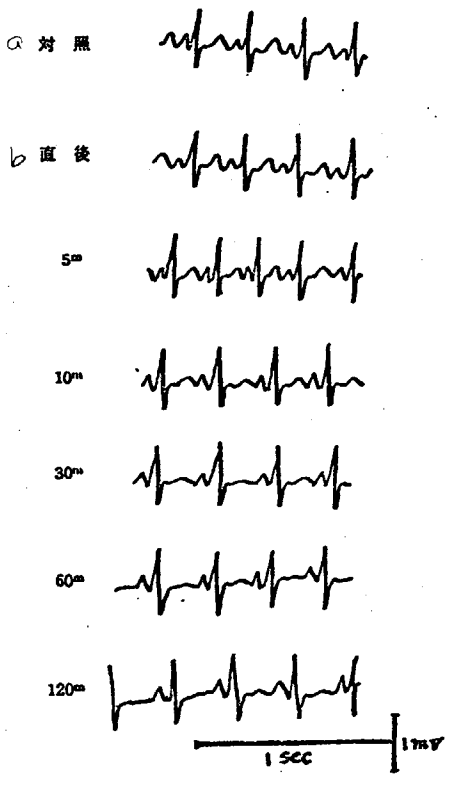
2×10^{-4}溶液では，5例中4例に薬液灌流直後から著明な振幅減少を示し，うち3例は薬液灌流中に，1例はRinger液交換ごにブロックが出現した。1例は振幅増大が一過性に現れたが，まもなく前値にもどり，同時にブロックが出現した。しかし多くの例がRinger液交換により5^m前後で回復した（第13図，1）。

b. クエン酸ナトリウム：4.8×10^{-7}溶液以下ではほとんど変化なく，4.8×10^{-6}溶液灌流ではやや振幅増大の傾向を示し，4.8×10^{-5}溶液では全例に振幅増大の傾向を示したが，1例はとくに著明で3^m後には前値の2.5倍となつた。しかしRinger液交換とともに回復し，10^m以内で前値にもどつた。これまでの濃度では搏動数ならびに緊張にほとんど変化をみとめなかつた（第12図，2）。

2.4×10^{-4}，4.8×10^{-4}溶液灌流ではいずれも振幅はかなり著明に減少したが，Ringer液交換後まもなく回復し，10^mころには増大するが，20^m前後にはほぼ前値に戻つた（第13図，2）。4.8×10^{-3}溶液ではこの傾向はさらに著明で，薬液灌流直後から心運動は拡張性停止をきたし，Ringer液を10数回交換して徐々に心運動が開始するが，ブロックが出現し緊張は著明に低下した。

c. 蓚酸ナトリウム：3.2×10^{-7}以下では変化なく，3.2×10^{-6}では5例中2例に変化なく，3例に薬液灌流直後軽度の振幅増大がみられ，5^m後Ringer液に交換してもさらに振幅増大が続いた。これは数回のRinger液交換により約20^m前後で回復した。また搏動数にはほとんど変化がなかつたが，緊張はわずかに低下の傾向を示した。3.2×10^{-5}溶液では3例中2例に軽度の振幅減少がみられたが，Ringer液交換と同時に復旧し，以後わずかに増幅の傾向を示した。他の1例は3.2×10^{-6}溶液と同様の増幅がみられたが，緊張にはほとんど変化がみられなかつた（第12図，3）。

3.2×10^{-4}溶液では灌流直後から著明な振幅減少が現われ，ブロックが出現する。搏動数も減少傾向を示すが，緊張にはほとんど変化がみられなかつた。Ringer液交換後大部分が比較的すみやかに回復し，そのご一時振幅は増大するが，数回のRinger液交換により復旧した（第13図，3）。3.2×10^{-3}では搏動は拡張性に停止し緊張は低下してRinger液交換によつて



第9図　蓚酸ナトリウムのウサギECGにたいする作用
Sod. Ox. 48mg/kg i.v.

も回復しなかつた。

2. カエル摘出心臓にたいする作用

実験材料および方法

体重22～30gのトノサマガエルを使用し，断頭脊髄破壊後背位に固定し，Straub 法の通法にしたがつて右大動脈を結紮，左大動脈弧よりカニューレを挿入して心室内に達せしめて結紮した。他の動静脈は一括して結紮し，心臓を摘出した。液圧は1.5cm H_2O とし，Ringer 液をときどき交換し，心運動が一定するのをまつて，心尖にセルフインをかけその運動をヘーベルをもつて煤煙紙上に描記させた。

薬液は八木式の場合と同様のものをもちいた。室温は23～25°C。

実験成績

a. 弗化ナトリウム：2×10^{-8} 溶液以下では心運動に全く変化なく，2×10^{-7} 溶液では薬液灌流中変化なく，Ringer 液交換後搏動数増加の傾向を示すことが多い。2×10^{-6} 溶液では5例中3例に変化なく，2例に軽度の一過性振幅増大がみられ，Ringer 液交換10～20^{m}後再び軽度の振幅増大，搏動数増加の傾向がみられた。2×10^{-5} 溶液では作用不定で，8例中2例に変化なく，4例に直後から著明な振幅減少と搏動数の増加がみられ，5^{m}後 Ringer 液交換により直後から振幅の回復はみられたが，搏動数は20^{m}ころ前値にもどつた。2例には灌流直後著明な振幅増大を示し，うち1例は Ringer 液交換10数回におよんでもさらに増大の傾向を示したが，他の1例は Ringer 液交換3^{m}後回復し，以後一過性のブロックをみた。搏動数，緊張にはほとんど変化がみられなかつた（第14図，1）。

2×10^{-4} 溶液では全例が灌流直後から振幅，搏動数の著明な減少をきたし，Ringer 液交換5^{m}後に振幅は回復したが，搏動数はまだ完全に前値に復さなかつた。緊張には著変なく，軽度の低下を示したものもあつた。0.2%では直後から心運動は拡張性停止をきたすが，直ちに Ringer 液で数回洗滌すると10～20^{m}で回復するが，そのご再び振幅減少，ブロックを出現することが多い（第15図，1）。

b. クエン酸ナトリウム：4.8×10^{-6} 溶液以下では変化なく，4.8×10^{-5} 溶液灌流では5例中2例に変化なく3例に振幅増大をみたが，Ringer 液交換後まもなく回復した。搏動数，緊張にはほとんど変化がなかつた（第14図，2）。4.8×10^{-4} 溶液では直後著明な一過性の振幅減少がみられたが，5^{m}ころには前値に復し，Ringer 液交換で一過性振幅増大を示したが，15^{m}ころには全例が復旧した。緊張には著変なく，搏動数も一般に軽度の増加傾向を示したにすぎない。4.8×10^{-3} 溶液では振幅減少著明で，3^{m}ころはブロックがあらわれ，5^{m}以内で多くの例が心運動の停止をみた。Ringer 液交換後も完全な回復はみられなかつた。緊張は低下を示すものもあつたが多くの例に著変をみなかつた（第15図，2）。

c. 蓚酸ナトリウム：3.2×10^{-7} 溶液灌流では作用不定で軽度の振幅増大または減少を示し確かではなかつた。しかし Ringer 液交換によりいずれも直ちに正常にもどつた。3.2×10^{-6} 溶液では全例に振幅の減少がみられた。Ringer 液交換により直ちに回復し，以後増大の傾向を示した。搏動数，緊張にはほとんど変化がみられなかつた。3.2×10^{-5} 溶液ではさらに著明な振幅減少がみられ，ブロックをみる例が多く（第14図，3），3.2×10^{-4}，3.2×10^{-3} 溶液ではさらにこの傾向は著明となり，振幅半減，緊張低下，搏動数の減少，および全例にブロックが現れ，Ringer 液交換で一時回復を示すが，再びブロックを現し正常運動を続けることはなかつた（第15図，3）。

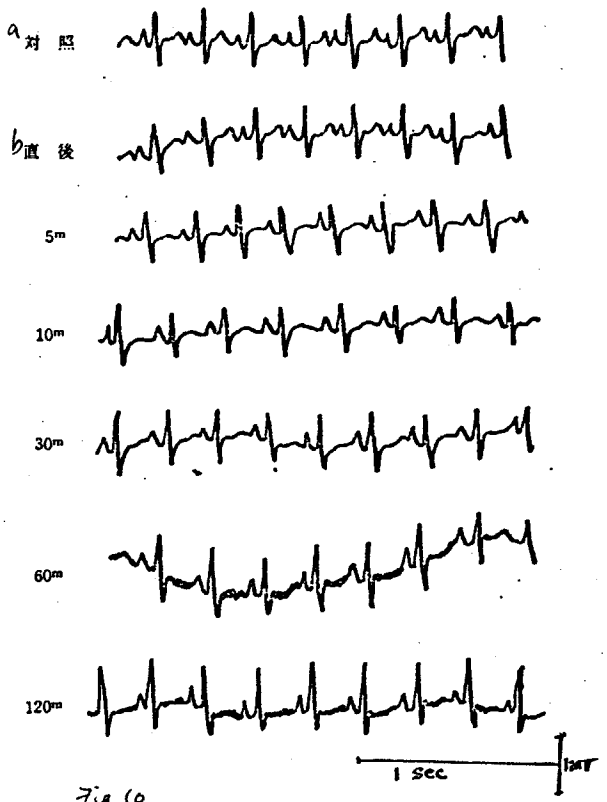
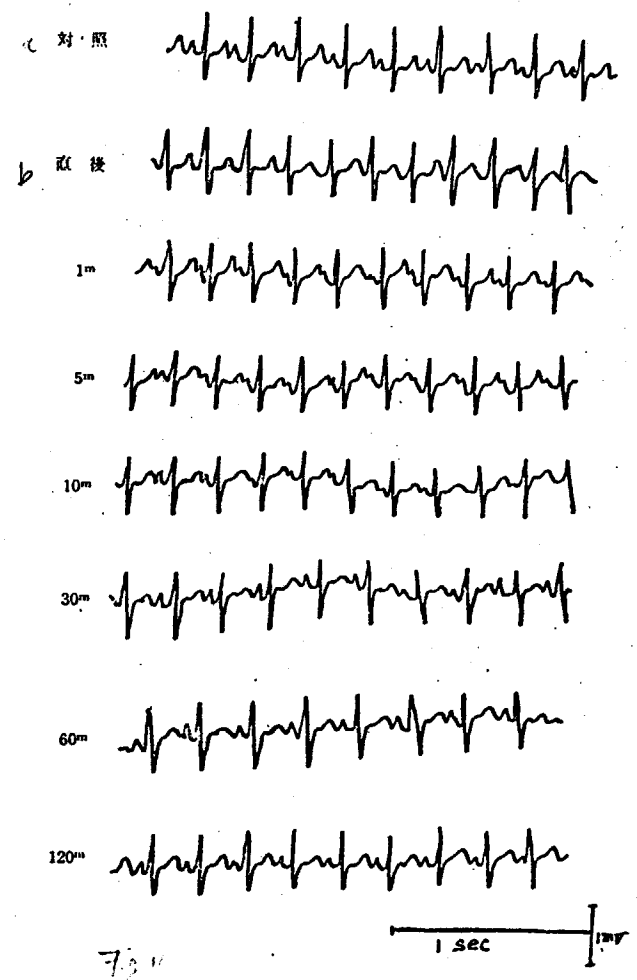


Fig 10
第10図　弗化ナトリウムのウサギＥＣＧにたいする作用
NaF 50mg/kg i.v.



第11図　クエン酸ナトリウムのウサギＥＣＧにたいする作用
Sod. Cit. 120mg/kg i.v.

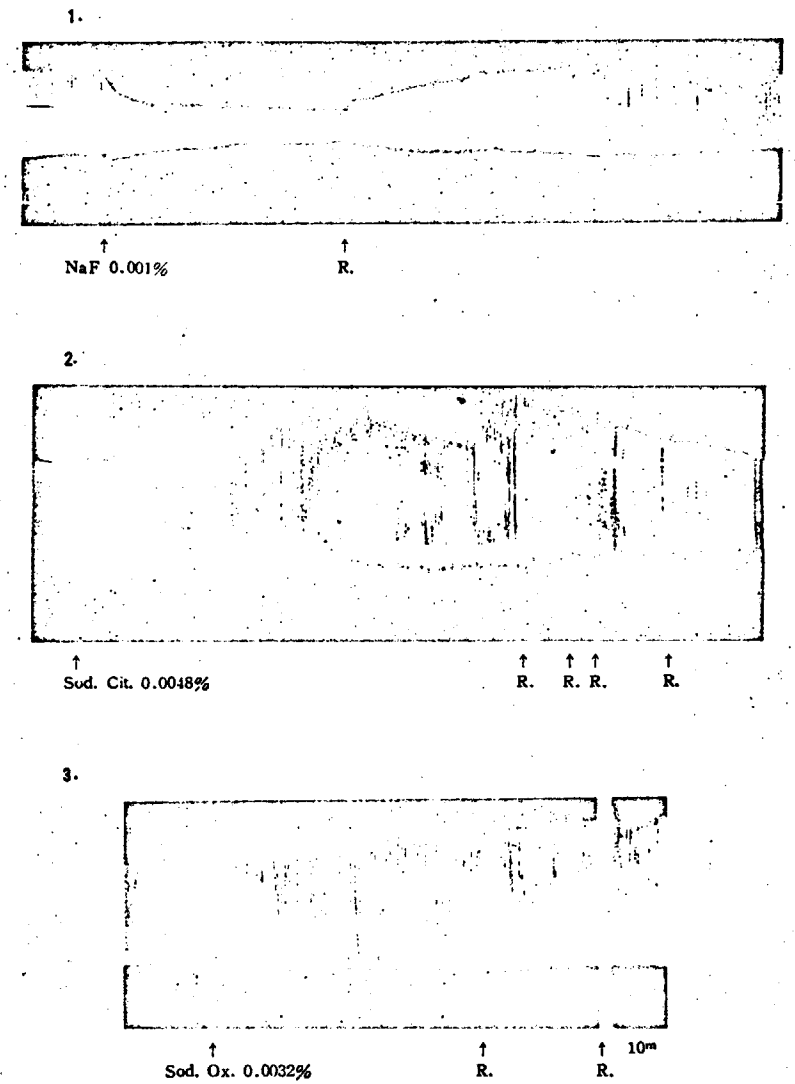
3. 各薬物へカルシウムイオン追加による影響

実験目的ならびに実験方法

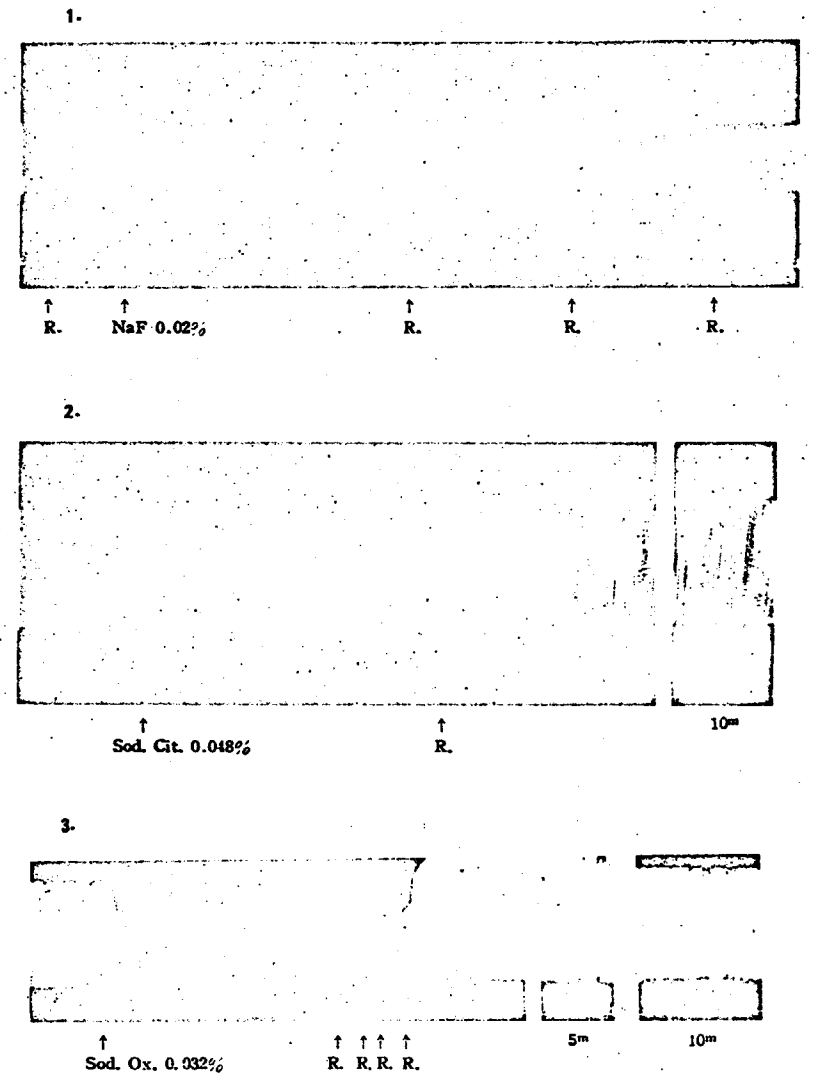
摘出カエルならびにヒキガエル心臓にたいする作用は，弗化ナトリウム0.02%，クエン酸ナトリウム0.048%，蓚酸ナトリウム0.032%前後の各溶液で，いずれも確実に心臓抑制効果があることがわかつたので，これらの薬物が Ringer 液に溶存しているさいにうばう Ca^{++} 量を追加して，これがヒキガエルの摘出心に及ぼす作用を，各薬物単独灌流時のそれと比較した。すなわち3薬物がこの濃度において，Ringer 液中のカルシウムをうばう計算量は約20 mg/dl であり，$CaCl_2\cdot2H_2O$ としてこれを補うためには約73 mg/dl が必要とされる。しかし $CaCl_2$ はきわめて潮解性がつよく，秤量中に理論上必要とされる量以下となるおそれがある。それゆえ念のため $CaCl_2\cdot2H_2O$ として約80mg/dl を各溶液中に追加した。ヒキガエル摘出心臓を標本として八木式灌流法によつて行つた。

実験成績

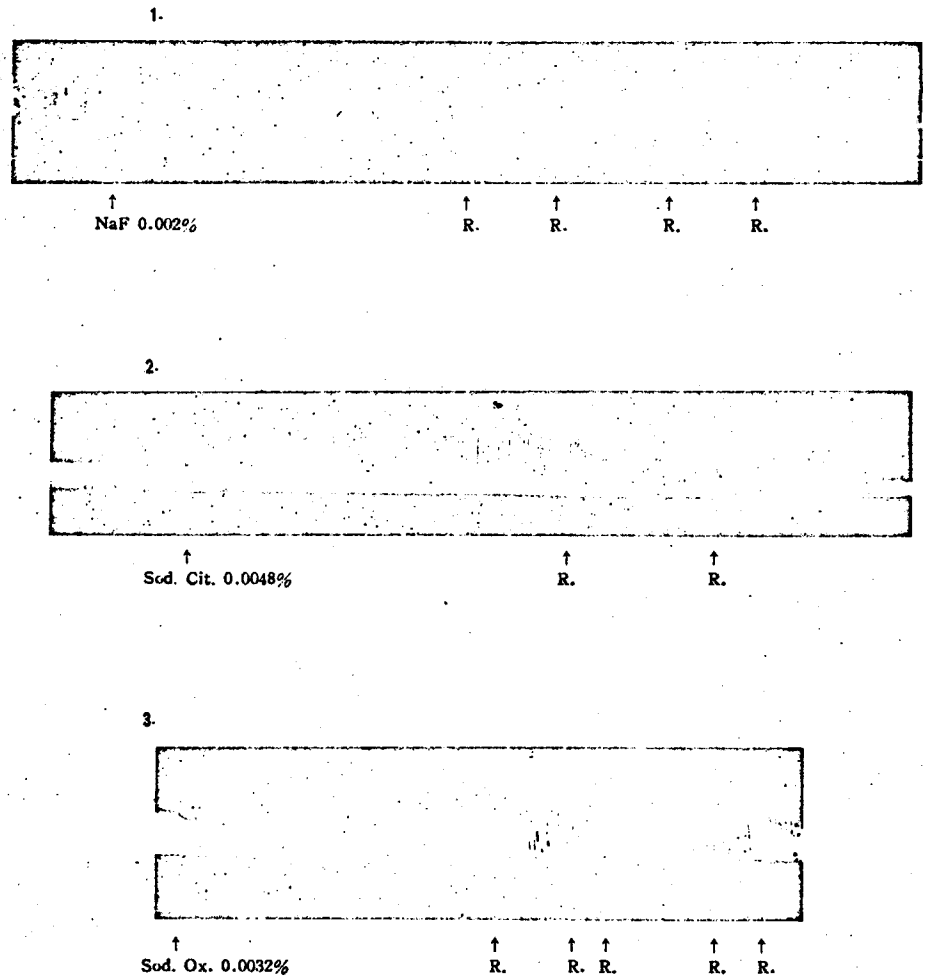
a. 弗化ナトリウム：Ca^{++} 加溶液（$CaCl_2$ 80mg/dl 加 NaF0.02%液）では，灌流後まもなく房室ブロックがあらわれ，搏動数の減少をきたした。しかし振幅



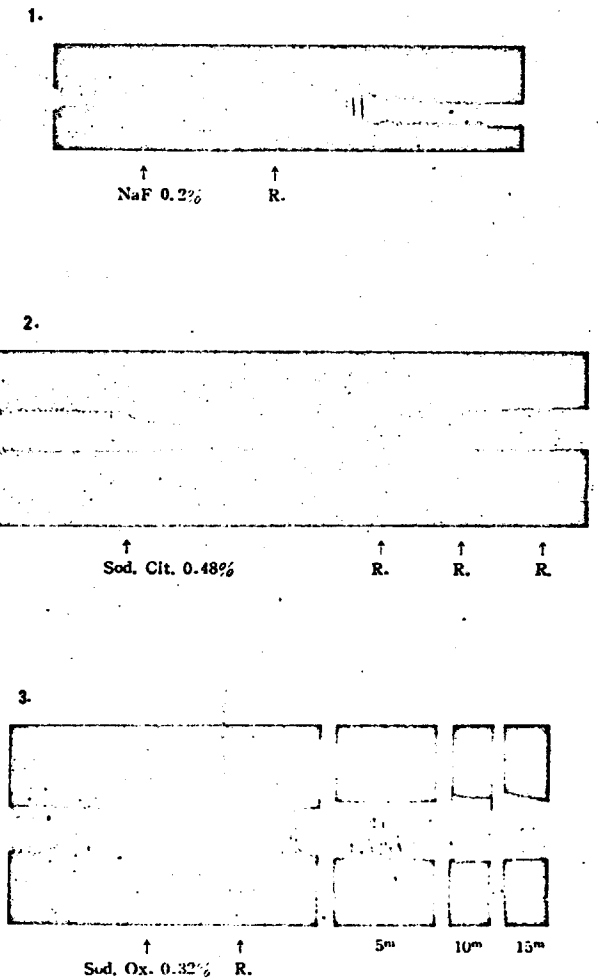
第12図　ヒキガエル摘出心にたいする弗化ナトリウム，クエン酸ナトリウム，蓚酸ナトリウムの作用（八木式灌流法）
R. は Ringer 液交換を示す



第13図　ヒキガエル摘出心にたいする弗化ナトリウム，クエン酸ナトリウム，蓚酸ナトリウムの作用（八木式灌流法）
R. は Ringgr 液交換を示す



第14図　摘出カエル心にたいする弗化ナトリウム，クエン酸ナトリウム，蓚酸ナトリウムの作用 (Straub 法)
R. は Ringer 液交換を示す



第15図　摘出カエル心にたいする弗化ナトリウム，クエン酸ナトリウム，蓚酸ナトリウムの作用 (Stranb 法)
R. は Ringer 液交換を示す

は弗化ナトリウム単独灌流時と異なりほとんど変化なかつた。さらに Ca^{++} 加溶液へ同量の $CaCl_2$ を追加しても振幅の増大のみが認められたにすぎず，伝導障害はなお続いた。5^m ご Ringer 交換によりまもなく回復し，以後は全く正常な搏動を続けた（第16図，1）。

b. クエン酸ナトリウム：$CaCl_2$ 80mg/dl 加 Sod. Cit. 0.048% 液の灌流直後に一過性の振幅減少を示したが，まもなく回復し以後はかえつて軽度増大の傾向がみられた。このさいさらに $CaCl_2$ を追加しても著変はなかつた。Ringer 交換とともに振幅は全く薬液灌流前にもどつた（第16図，2）。

c. 蓚酸ナトリウム：クエン酸ナトリウムとほぼ同様に，$CaCl_2$ 80mg/dl 加 Sod. Ox. 0.032% 液の灌流直後一過性の振幅減少と緊張低下がみられたがまもなく回復し，そのごさら $CaCl_2$ を追加すると，緊張はかえつて一過性に上昇するがまもなく正常にもどつた（第16図，3）。

Ⅱ　ウサギの血圧呼吸ならびに心動にたいする作用

実験材料ならびに実験方法

体重 2.5kg 前後の雄性成熟ウサギを，10% Urethane 1g/kg あるいは Nembutal 40mg/kg の腹腔内注射によつて麻酔してから，背位に固定し，剪のごとく開胸して，心搏動は Cushny の Myocardiograph を用い，血圧は頸動脈にカニューレを挿入し，水銀マノメーターに連結した。また呼吸は気管カニューレによつて Marey のタンブールに連結し，同時に煤煙紙上に描記させた。

薬物はいずれも生理食塩水溶液とし，耳介静脈より静かに注射した。

実験成績

a. 弗化ナトリウム静注：5mg/kg 以下の量では呼吸，血圧心動に変化なく，20～30mg/kg では呼吸は一過性興奮を示し，振幅の増大が比較的著明に現れたが，呼吸数にはあまり変化がなかつた。10^m ころには呼吸振幅はかえつて小となり，20^m 前後で旧に復した。血圧は注射後まもなく下降しはじめ，15^m 前後で約 20mmHg 下降し，以後しだいに回復して 30^m 前後ではほぼ旧に復した。心動振幅は注射中一過性の軽度減少を示すが，血圧下降にともなつて増大を示した。緊張にはほとんど変化がみられなかつた（第17図）。

50mg/kg i. v. では注射中から直後にかけて呼吸数は増加し，著明な振幅の増大（約3倍）がみられたが，そ[illegible]不整となり数はかえつて減少した。このさいしば[illegible]涎，流涙がみられ，暴れることが多かつた。呼吸振幅は20～30^m 後にほぼ前値にもどるが，なかには 2^h 以上経過しても旧に復さぬものもあつた。血圧は注射終了直後より著明に下降し，10～30^m 後で最低（約 50mmHg 下降）となり，以後 1^h ころまで持続することが多い。心動は注射直後一過性の振幅減少がみられたが，まもなく回復した。しかし血圧下降にともなつて再び減少し，90^m 前後でほぼ回復した。緊張にはほとんど変化がみられなかつた（第20図）。またこの量では5例中2例が激しい呼吸興奮のち麻痺をきたし人工呼吸によつても回復せず，痙攣，眼球突出，瞳孔散大，流涎，流涙を呈し，ときに叫声を発して，注射後 10^m 前後で心運動の拡張性停止をきたして死亡した。

b. クエン酸ナトリウム静注：20mg/kg 以下の量でほほとんど変化なく，48mg/kg では呼吸は一過性の興奮を示し振幅，呼吸数の増加が著明に現れる。そのご一時減少するが，20^m 前後にはほぼ前値にもどつた。血圧は注射後ただちに下降（約 20mmHg）するが 10^m 前後で前値に復し，以後はかえつて上昇の傾向を示す。心動は注射と同時に著明な一過性の緊張低下と振幅減少がみられるが，10^m 前後で回復し，以後やや増大の傾向をみることが多い。72mg/kg の場合もほぼこれと同様の変化がみられた（第18図）。

120mg/kg ではさらに著明な血圧下降（約 50mmHg）がみられ，呼吸興奮も著明で振幅は約5倍となり，心動振幅は著明に減少し，緊張著しく低下する。しかしまもなく血圧は回復を示し，約30^m 前後にはほとんど前値に復し，以後はかえつて上昇の傾向を示すことは小量注射の場合と同様であつた。呼吸はそのご一時停止することが多いが，2～3回の人工呼吸でまもなく回復し，以後再び呼吸幅は4～5倍に増大し，数もしだいに多くなり，注射後 5^m 前後には前値の約2倍に増加した。呼吸数増加とともに振幅はかえつて小となるが，30～40^m でほぼ回復した。心運動は血圧の回復に伴つて 30^m 前後でほぼ回復した（第21図）。この量ではかなりゆつくり注射をしても流涎，流涙がみられ，口唇をなめて四肢を動かし猛烈に暴れ，けいれんもみられることがあるが，いずれも一過性で，多くの例がよくこれに耐え実験を終つた。しかし注射を迅速かつ急激に行うときは，激しい呼吸興奮のち麻痺が長く続き，叫声を発し，心臓の拡張性停止をきたして死亡した例もあつた。

c. 蓚酸ナトリウム静注：8mg/kg 以下の量ではほとんど変化はみられず，16mg/kg では一過性軽度の呼吸興奮後抑制がみられ，血圧は徐々に下降して，注射後 10^m ころは 10～20mmHg 下降し，以後 10～20[illegible]

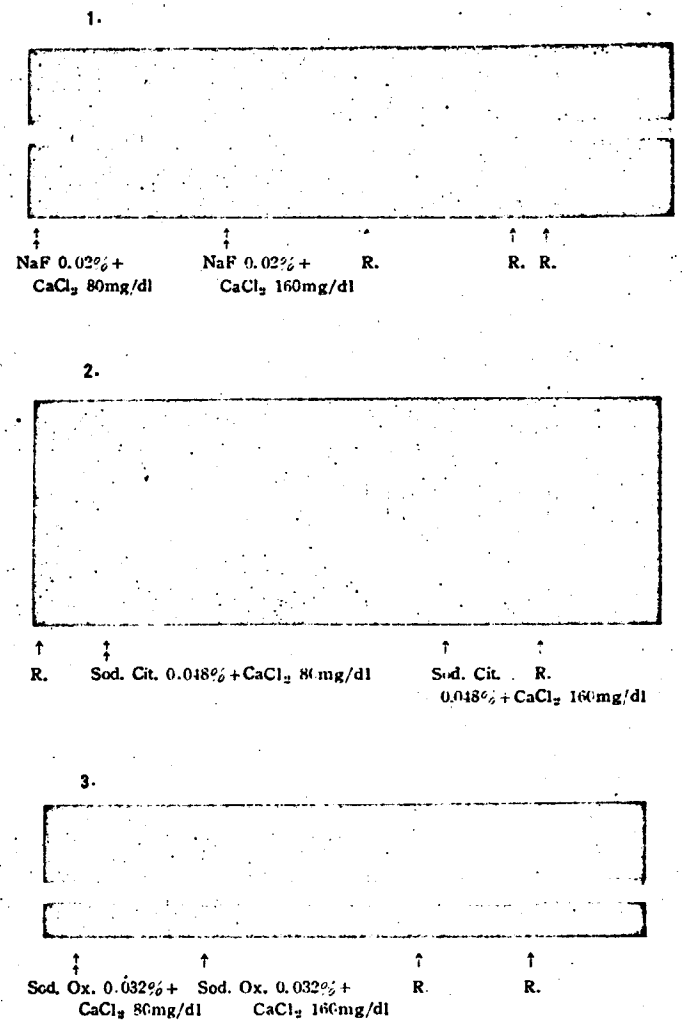
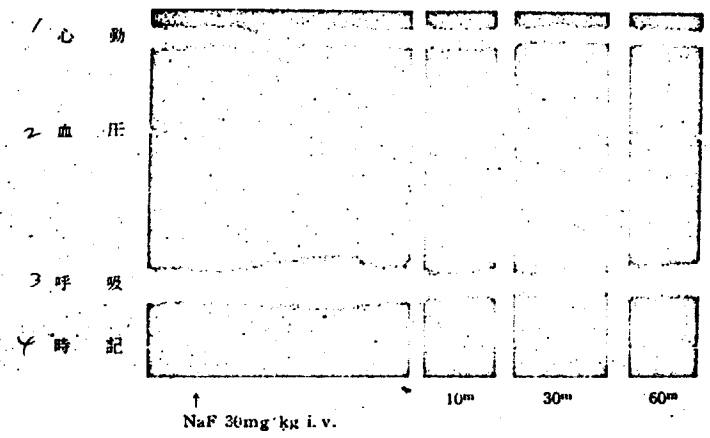
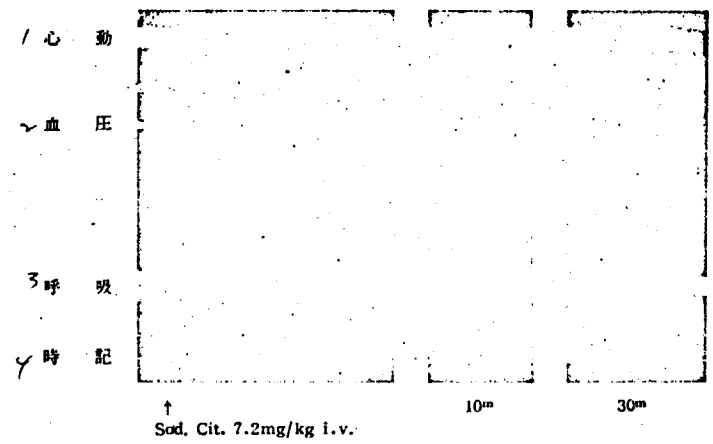


Fig 16 第16図　ヒキガエル摘出心にたいする弗化ナトリウム，クエン酸ナトリウム，蓚酸ナトリウムの作用と Ca^{++} 添加の影響（八木式灌流法）
1. 弗化ナトリウムと Ca^{++}
2. クエン酸ナトリウムと Ca^{++}
3. 蓚酸ナトリウムと Ca^{++}
R. は Ringer 液交換を示す



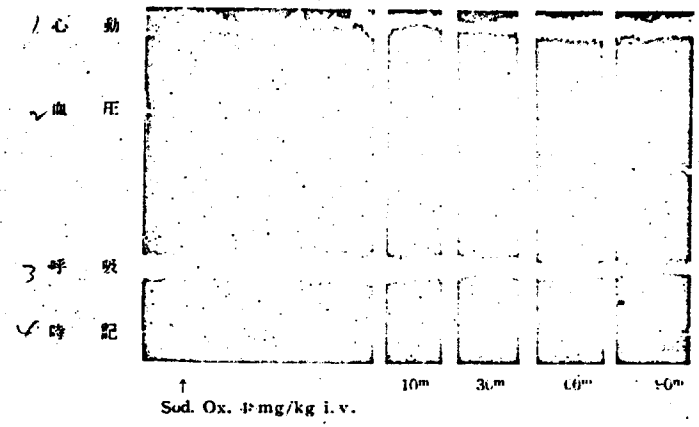
第17図　ウサギの心動・血圧・呼吸にたいする弗化ナトリウムの作用

Fig 17



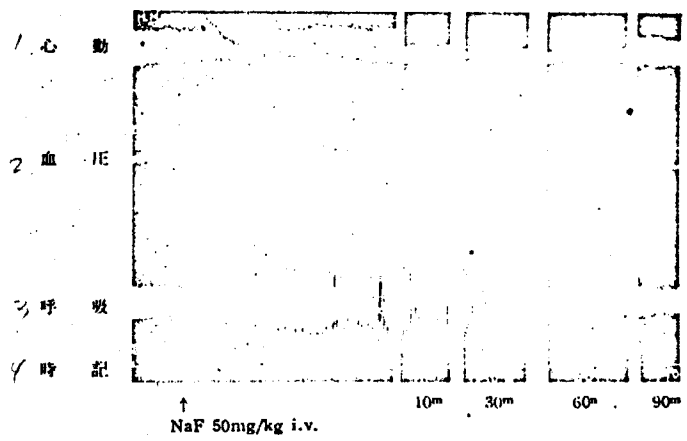
第18図　ウサギの心動・血圧・呼吸にたいするクエン酸ナトリウムの作用

Fig 18



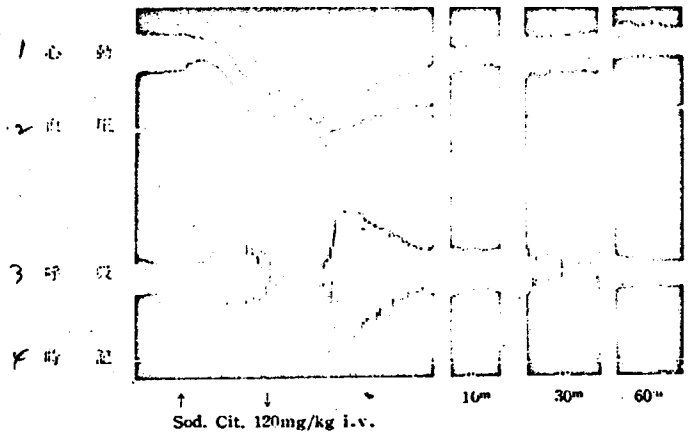
第19図　ウサギの心動・血圧・呼吸にたいする蓚酸ナトリウムの作用

Fig 19



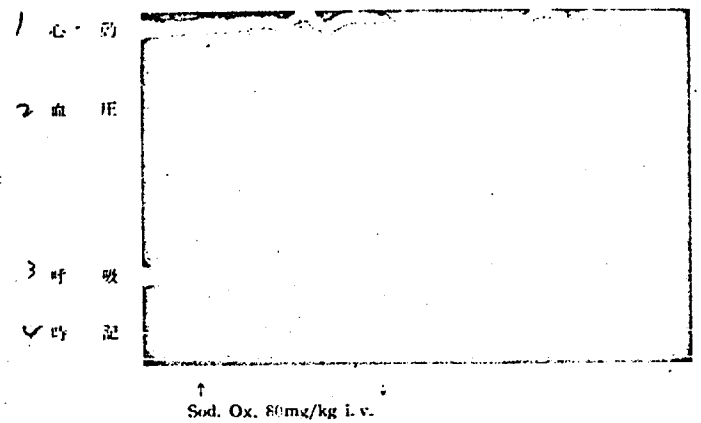
第20図　ウサギの心動・血圧・呼吸にたいする弗化ナトリウムの作用

Fig 20



第21図　ウサギの心動・血圧・呼吸にたいするクエン酸ナトリウムの作用

Fig. 21



第22図　ウサギの心動・血圧・呼吸にたいする蓚酸ナトリウムの作用

Fig. 22

持続する。心動は注射直後軽度の緊張低下と振幅の減少がみられるが，注射後40～50^{m}で血圧回復にともなつて回復し，以後はかえつて増幅の傾向を示す。

32～48mg/kg では注射速度が速いと，直後はげしい牙関緊急，流涎，流涙をきたし，呼吸数は増加して不整となり，血圧は時間とともに下降し，ついには呼吸麻痺をきたし，心運動は拡張性停止をきたして死亡する。このさい人工呼吸によつても回復しなかつた。注射をきわめてゆつくりと行えば（3.2～4.8mg/kg/min 位）血圧は徐々に下降し，50～60^{m}で最低（30～40mmHg 下降）となり，90～120^{m}でも前値に回復しないことが多い。呼吸振幅は直後増大して約2倍くらいになり呼吸数はかえつて減少傾向を示す。20～30^{m}で振幅が小となるにつれ数は増加の傾向を示す。心動は直後から抑制され，緊張低下をきたすが，40～50^{m}ごには徐々に振幅増大し，90^{m}前後で回復する（第19図）。

80mg/kg では注射速度をさらにゆつくり行つても，血圧は下降を続け，呼吸興奮のち麻痺に陥り，心運動は拡張性停止をきたして死亡する例が多かつた（第22図）。

Ⅳ　血管中枢にたいする作用

実験材料及び実験方法

神田ら[8]による新実験法によつておこなつた。本実験法は実験操作が比較的簡易なこと，動物（ウサギ）を腹位に固定するので比較的自然体位に近いこと，麻酔をしないこと，上半身の皮膚血管（耳介血管）を使用すること，末梢血管作用を容易に除去しうること，心臓機能変化の影響なしに純粋の中枢性血管作用を測定しうることなどの点で従来の中枢血管作用実験よりもすぐれているとおもわれるのでこの方法を応用した。

すなわち無麻酔ウサギを腹位に固定し，耳の基部において神経，動静脈を露出分離し，これらを残して耳の基部をカン子で挟んで切断した。のち静脈は結紮切断し，動脈にカニューレを挿入して Mariotte 壜よりの Ringer 液を灌流し，静脈より流出させ，滴数が一定してから薬物を反対側の耳介静脈より注射してその作用を観察した。このさいとくに神経の保護に注意し，その他の注意事項は神田らの報告に従つた。

実験成績

a.　弗化ナトリウム：10mg/kg 静注では著変なく，30mg/kg 静注で注射後3^{m}ころから滴数増加を示し，5^{m}ころには50%前後の増加を示したが，10^{m}前後ではほぼ回復した。50mg/kg 静注ではさらに著明な滴数増加がみられ，20^{m}ころまで続いた。

b.　クエン酸ナトリウム：24mg/kg 静注では変化なく，72mg/kg 静注で注射直後一過性軽度の滴数増加がみられたが，5^{m}前後で回復した。

c.　蓚酸ナトリウム：16mg/kg i.v.では変化なく，48mg/kg i.v.では注射後5^{m}ころから軽度の滴数増加がみられ，40～50^{m}持続した（以上第23図）。

Ⅴ　末梢血管にたいする作用

1. ヒキガエル後肢血管にたいする作用

実験材料及び方法

Läwen-Trendelenburg 法にしたがい，薬液は生理食塩水または Ringer 溶液とし，内容500ml の Mariotte 壜2個のうち，一方には Ringer 液又は生理食塩水を，他方には各種濃度の薬液を充し，Y字管によつて連結し，活栓を切り換えて灌流を行つた。滴下数が一定するのをまつて（20～40滴/min），薬液に切り換え灌流し，一定時間後再びもとの灌流液にもどして前後の変化を観察記録した。

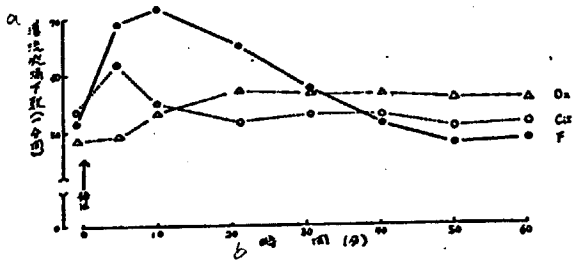


Fig. 23　第23図　ウサギ耳介血管灌流（神田法：血管中枢作用）にたいする Sod. F., Sod. Cit., Sod. Ox. の影響
F：弗化ナトリウム 30mg/kg i.v.
Cit：クエン酸ナトリウム 72mg/kg i.v.
Ox：蓚酸ナトリウム 48mg/kg i.v.

実験成績

a.　弗化ナトリウム：10^{-5}溶液以下ではほとんど作用なく，10^{-4}溶液10^{m}間の灌流で滴下数は約半減し，著明な血管収縮をみとめた。しかし Ringer 液に交換すると直ちに回復し，約10^{m}前後で正常にもどつた。2×10^{-4}溶液では10^{m}後同様に滴数は半減するが，Ringer 液に交換してもわずかに回復の傾向を示すのみで，前値にはもどらなかつた。5×10^{-4}ではこの傾向がさらに

著明で，10^{m}後は $^{1}/_{4}$～$^{1}/_{2}$ に滴数減少し，Ringer 液に切り換えた後も全く回復の傾向がみられず，さらに減少して15～20^{m}ごにはほとんど停止した。

b. クエン酸ナトリウム：4.8×10^{-5} 溶液から滴数の軽度増加がみられた。Ringer 液切り換え後ただちに回復し，以後やや減少傾向を示すことが多い。しかし濃度を増して4.8% 溶液としてもとくにいちじるしい作用の相違はみられず，灌流中軽度の滴数増加を示したが，Ringer 液切り換え後 30～40^{m} では回復しなかつた。

c. 蓚酸ナトリウム：3.2×10^{-5}溶液では増減不定で，3.2×10^{-4} 以上では軽度の滴数増加をみるにすぎず，Ringer 液交換後まもなく前値に復することもあるが，多くはそのまま持続した。3.2% 溶液では溶解不完全で，滴数減少はみられたが確かではなかつた（第24図）。

2．ウサギの摘出耳介血管にたいする作用

実験材料及び実験方法

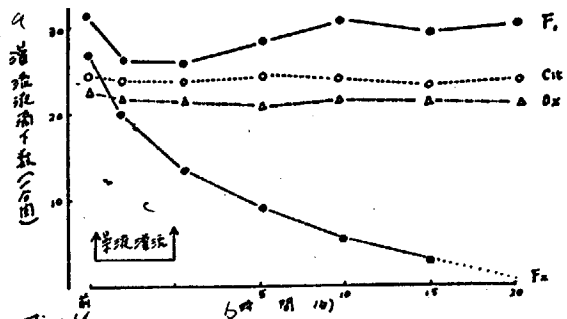
Krawkow-Pissmski 法にしたがい，実験した。ウサギ耳介は分離後12～24^{h}，4°C 氷室に保存したものを使用した。灌流方法はヒキガエル後肢血管灌流と同様の方法で行つた。

実験成績

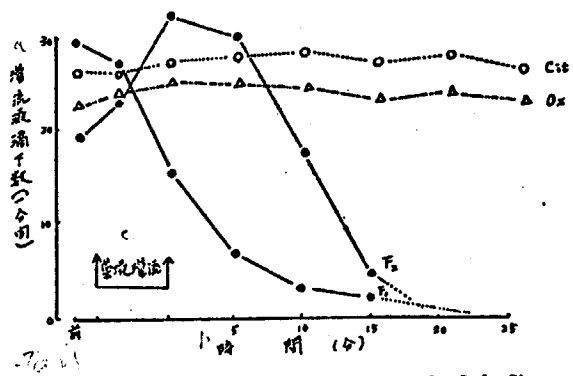
a. 弗化ナトリウム：10^{-5}溶液ではわずかに滴数増加の傾向を示すが著変はなかつた。10^{-4} 溶液では毎常軽度の滴数減少がみられた。しかし灌流時間を長く（30^{m}）しても滴数減少は進まず，Ringer 液交換とともに直ちに回復する。2×10^{-4} 溶液では著明な滴数減少を示し，灌流時間を延長するとさらに著明となるが，Ringer 液交換後は10^{m}前後で回復しはじめ，30^{m}前後ではほぼ前値にもどる。5×10^{-4} 溶液ではさらに強い収縮を示し，Ringer 液に切り換えても回復することはほとんどない。

しかし1%では灌流中はかえつて[illegible]く，Ringer 液に交換するとまもなく減少し，以後時間とともに前値以下となる。2%では著明な滴数増加を示すことが多く，なかにはほとんど算定不能なほどの増加を示した。しかし Ringer 液交換により毎常滴数は減少し，20^{m}ころには全く停止することが多い。

b. クエン酸ナトリウム 2.4×10^{-4} ていどから軽度の滴数増加がみられ，4.8×10^{-3} 溶液でも著明な作用はなく，軽度の滴数増加がみられるにすぎなかつた。0.12%溶液ではかえつて作用なく，Ringer 液交換後やゝ増加傾向がみられた。2.4～4.8%溶液ではきわめ



第24図　ヒキガエル後肢血管灌流にたいする Sod. F., Sod. Cit., Sod. Ox. の作用
F_1：弗化ナトリウム 0.01 %
F_2：弗化ナトリウム 0.05 %
Cit：クエン酸ナトリウム 0.024%
Ox：蓚酸ナトリウム 0.016%



第25図　ウサギ耳介血管灌流にたいする Sod. F., Sod. Cit., Sod. Ox. の作用
F_1：弗化ナトリウム 0.05%
F_2：弗化ナトリウム 2.0 %
Cit：クエン酸ナトリウム 0.12%
Ox：蓚酸ナトリウム 0.08%

て軽度の滴数減少を示すが，Ringer 交換後まもなく回復し以後軽度の増加傾向を示した。

c. 蓚酸ナトリウム：3.2×10^{-4} 溶液では灌流中きわめて軽度の滴数増加を示すが，Ringer 液交換後まもなく回復する。3.2×10^{-3} 溶液でもほぼ同程度の作用を示した。1.6%以上は軽度の滴数減少がみられ，Ringer 交換によつて回復するが，まれに減少が続いたこともあつた（第25図）。

Ⅵ　ウサギの利尿にたいする作用

実験材料及び方法

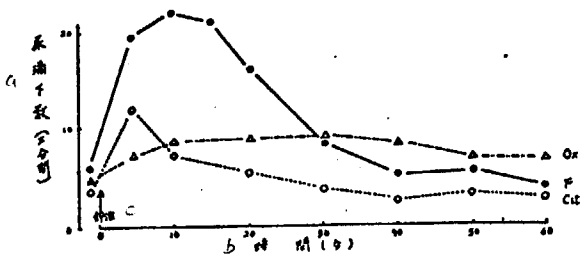
体重2～3kg の雄性成熟ウサギに，あらかじめ100 ml の水を胃管によつて投与したのち，Urethane または Nembutal 麻酔でウサギを背位に固定し，輸尿管カニューレ法によつて実験した。Urethane 麻酔の場合は，いわゆる Urethane diuresis の時期をさけて，尿量の一定となるのをまつて，耳介静脈よりゆつくり薬液を注射した。使用薬液は弗化ナトリウム2%，クエン酸ナトリウム4.8% 及び蓚酸ナトリウム 1.6% の生理食塩水溶液とした。

実験成績

a. 弗化ナトリウム静注：5mg/kg では著明な作用なく，10～20mg/kg では著明な尿量増加がみられ，10^{m}前後には注射前の 6～7倍に達し，20^{m}ころからしだいに減少するが，30^{m}でも前値にもどらないことが多かつた。50mg/kg では注射直後一過性に 3～4 倍の尿量増加をみるが，まもなく減少して前値以下となる。

b. クエン酸ナトリウム：12mg/kg 以下の量ではほとんど作用なく，24～48mg/kg では注射直後から尿量増加して 5～10^{m}で前値の 3～4 倍に達し，30^{m}前後で前値に復した。120mg/kg では注射直後暴れ，尿量は一過性に $^{1}/_{3}$～$^{1}/_{2}$ 減少するが，10^{m}前後呼吸鎮静とともに再び前値に戻つた。しかし以後の観察でも尿量増加の傾向はみられなかつた。

c. 蓚酸ナトリウム：16mg/kg では一過性の軽度の尿量増加を示したが，10～20^{m}で前値にもどり，以後はやや減少の傾向がみられた。32mg/kg では注射直後から 3 倍程度の尿量増加を示し，注射後 10～20^{m}からゆるやかに減少して，1^{h}前後でほぼ前値にもどつた。80mg/kg では，血圧・呼吸の項で述べたような症状が現れ，ほとんど測定不能であつた。1 例において，実験中股静脈より Ringer 液の点滴静注をしながら行つたものについては，注射後10^{m}前後で尿量は半減し，1^{h}ころ回復した（以上第26図）。



第26図　Sod. F., Sod. Cit., Sod. Ox. のウサギ利尿試験
（輸尿管カニューレ法）
F：弗化ナトリウム 20mg/kg i.v.
Cit：クエン酸ナトリウム 48mg/kg i.v.
Ox：蓚酸ナトリウム 32mg/kg i.v.

Ⅶ　ウサギの腸管にたいする作用

A．生体内腸管にたいする作用

実験方法

体重 2～3kg の雄性成熟ウサギを Nembutal 40 mg/kg 腹腔内注射により麻酔し，背位に固定して開腹し，Trendelenburg 法にしたがい小腸運動をヘーベルをもつて煤煙紙上に描記させた。実験中ウサギが暴れたために位置や緊張に変化をきたさぬように，小腸固定器の装着に注意した。薬物は耳介静脈内へ注射した。

実験成績

a. 弗化ナトリウム静注：5mg/kg では軽度の振幅増大がみられたにすぎないが，10mg/kg では著明な振幅の増大と軽度の律動数の減少が20～30^{m}続いた（第27図）。30mg/kg では注射直後一過性の緊張上昇がみられるが，まなく旧位にもどり，10^{m}ころから再びゆるやかに緊張上昇の傾向がみられた。振幅には著変なく運動数は軽度増加し，20^{m}後ころから再びわずかに振幅の抑制傾向を示した。

b. クエン酸ナトリウム静注：12 mg/kg ではほとんど作用なく，24 mg/kg では軽度の一過性緊張上昇と律動数増加，振幅減少がみられるが，まもなく回復する（第28図）。72mg/kg では直後一過性緊張上昇後，一過性に緊張低下を示しまもなく旧位に復する。振幅，運動数には著変はみられなかつた。

c. 蓚酸ナトリウム静注：8mg/kg ではほとんど作用なく，16mg/kg 静注では一過性軽度の緊張上昇と振幅[illegible]傾向を示すが，律動数は

や減少する。しかしいずれも注射後15ᵐ前後には旧に復する（第29図）。48mg/kg では注射直後から緊張は上昇を示すが，まもなく前値より低下し振幅，運動数ともに減少して30ᵐ以上経過しても回復はみられなかつた。

B. 摘出腸管にたいする作用

実験方法

ウサギを撲殺後，ただちに小腸を摘出して冷 Tyrode 液中に保存し，または無処置生体のまま背位に固定し，開腹して，いずれも1.5〜2.0cm に小腸を切断し Magnus 法にしたがつてその運動を煤煙紙上に描記させた。薬物は各濃度の Tyrode 溶液として調製し，恒温槽中に貯蔵して，これと Tyrode 液とを交換してその変化を観察した。灌流液はつねに 30ml とし，液温は 38°C とした。

実験成績

a. 弗化ナトリウム：10^{-6} 溶液では灌流直後からかなり著明な振幅増大がみられ（2倍前後），以後 Tyrode 液交換によつてもさらに増大傾向がみられた。緊張は上昇傾向を示すが，律動数にはほとんど変化がなかつた。10^{-5} 溶液では灌流直後振幅は逆に抑制され，緊張は一過性に低下し，のち振幅増大とともに上昇の傾向を示すが，Tyrode 液交換により再び振幅減少，緊張低下を示す。しかしいずれも一過性でもまなく回復し，のち振幅は大となり，緊張もさらに上昇傾向を示した（第30図，1）。10^{-3} 溶液では律動は全く停止し，緊張は著明に上昇する。Tyrode 液に交換しても律動は開始せず，緊張のみがさらに上昇した（第30図，2）。

b. クエン酸ナトリウム：2.4×10^{-6} 及び 2.4×10^{-5} 溶液では，直後一過性の緊張低下と振幅減少がみられるがまもなく回復し，のち著明な振幅増大と緊張上昇がみられ（第30図，3），2×10^{-4} 溶液では振幅減少して前値の½程度になるが，Tyrode 液交換とともに回復を示し，1ᵐ 以内で前値にもどるが，以後さらに増大し2倍以上の増幅を示す。緊張は灌流中やや低下の傾向を示すことが多いが，Tyrode 液交換後はつねに上昇する。2.4×10^{-3} 溶液では律動全く停止し，緊張低下がみられた。Tyrode 液に交換すると緊張はさらに低下したが，徐々に回復し，運動も再び開始し，3ᵐ 後緊張は前値より高くなり，振幅も大となつた（第30図，4）。

c. 蓚酸ナトリウム：1.6×10^{-6}〜1.6×10^{-5} 溶液で，著明な振幅の増大と緊張上昇がみられ，1.6×10^{-4} 溶液で[illegible]流直後から振幅は減少し，Tyrode 液交換後は[illegible]回復し，前値より増大する。緊張は上昇傾向を示す（第30図，5）。しかし，1.6×10^{-3}，1.6% 溶液では律動は全く停止し，緊張著明に上昇した。Tyrode 液交換によるも運動は開始せず，緊張は低下を示すことが多かつた（第30図，6）。

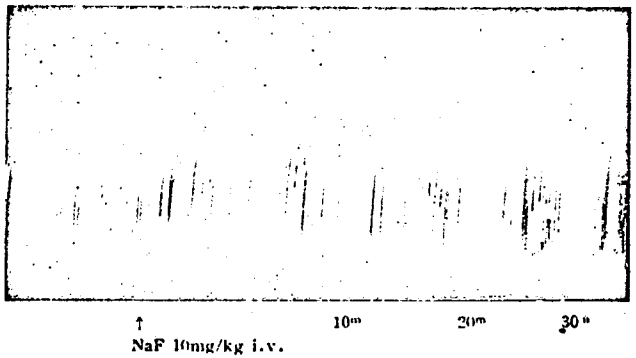
VII ウサギの血清総カルシウム，透析性カルシウム，総マグネシウムおよび無機燐の変動におよぼす作用

実験材料及び実験方法

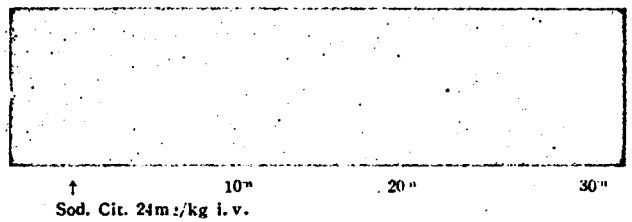
体重2.5kg 前後の成熟雄性ウサギを使用し，実験前夜から絶食せしめて実験した。薬物は生理食塩水溶液として耳介静脈よりしずかに注射した。

採血：心臓より穿刺により直接1回 2ml づつとり，注射前のものを対照用として，注射後20ᵐ，1ʰ および4ʰ 後，またときには24ʰ 後にも採血し，有栓遠沈管に入れ，凝血後遠沈し，血清を分離して定量した。実験中の室温は 17〜22°C であつた。

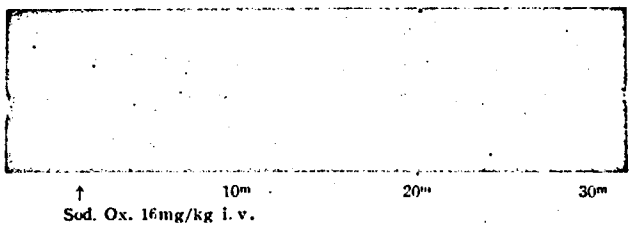
定量法：血清総カルシウム（以下血清 Ca と略記）および血清総マグネシウム（以下血清 Mg と略記）は柳沢法[56]により行つた。血清透析性カルシウム（以下血清 $Ca^{\cdot\cdot}$ と略記）は柳沢法によると，極端に時間に制限され（試薬を入れて測定まで3ᵐ間），ときとして実験誤差が大きくなり易いので，鈴木[57]により改良された方法で行つた。すなわち血清 0.2ml を有栓遠沈管にとり，4%蓚酸アンモニウム 0.8ml を加えて1ᵐ間充分混和振盪し，これを 1,000r.p.m. 1ᵐ間遠沈のち直ちに上澄液 0.5ml を試験管にとり，以後は柳沢法と全く同様の操作によつて試薬を加え測定した。この方法によると蓚酸アンモニウムと結合した透析性カルシウムが沈澱して残るので，測定されるカルシウムは結合型カルシウムであるから，時間的にも極めて安定した値がえられる。血清 $Ca^{\cdot\cdot}$ は血清 Ca からこの結合型 Ca 量を差引いた値をもつて表わした。血清無機燐（以下血清 P と略記）は Fiske & Subbarow 法[58]により，いずれも東京光電式光度計 7−A 型を用いて測定した。なお個々の対照とは別に，生理食塩水 2ml/kg を静注して，実験群と同様の採血方式で，採血量を1回 2ml および 5ml として，その血清 Ca，血清 $Ca^{\cdot\cdot}$，血清 Mg および血清 P の変動におよぼす採血量の影響をしらべた。第1表はその成績を示す（第1表）。すなわち1回の採血量が 2ml で，1ʰ 毎に3〜4回の採血を行つたていどではほとんど血清電解質に認めるほどの変動はないが，5ml では血清 Ca および血清 $Ca^{\cdot\cdot}$ が時間とともに減少し，血清 Mg および血清 P は逆相関的に上昇の傾向を示し，明らかな変動[illegible]みられた。したがつて本実験では1回採血量を 2ml[illegible]



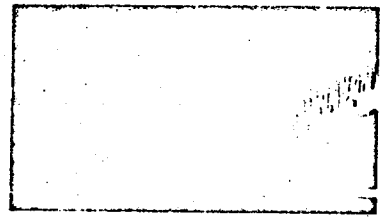
第27図　ウサギ生体内腸管にたいする弗化ナトリウムの作用 (Trendelenburg 法)



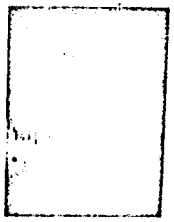
第28図　ウサギ生体内腸管にたいするクエン酸ナトリウムの作用



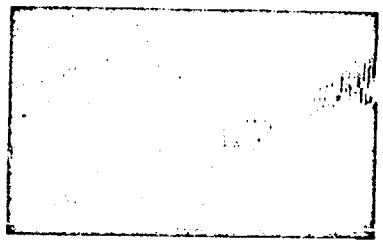
第29図　ウサギ生体内腸管にたいする蓚酸ナトリウムの作用



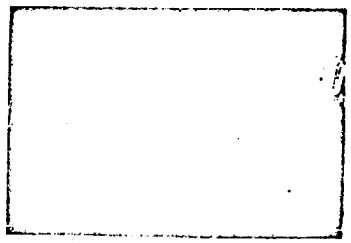
1. 弗化ナトリウム



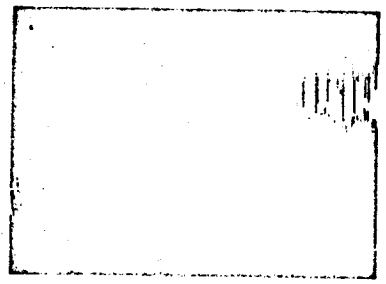
2. 弗化ナトリウム



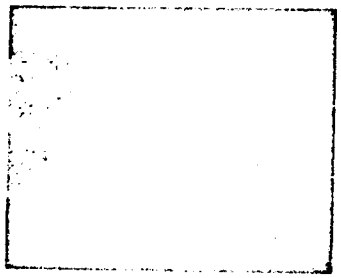
3. クエン酸ナトリウム



4. クエン酸ナトリウム



5. 蓚酸ナトリウム



6. 蓚酸ナトリウム

第30図　ウサギ摘出腸管にたいする弗化ナトリウム，クエン酸ナトリウム，蓚酸ナトリウムの作用　(Magnus 法)

第1表　採血量による血清電解質の変動

ウサギ No.	採血量	時間	総Ca	透析性Ca	総Mg	無機P
No. 2 2.5kg ♂	毎回 2ml	対照	14.0	6.1	1.9	6.5
		20m	14.0	6.0	1.8	6.5
		1h	13.9	6.1	1.9	6.7
		4h	14.1	6.2	1.8	6.4
		24h	14.2	6.1	1.8	6.6
No. 3 3.1kg ♂	毎回 2ml	対照	15.1	6.5	2.1	6.9
		20m	14.9	6.5	2.0	6.8
		1h	15.0	6.3	1.9	6.9
		4h	15.0	6.2	2.1	6.7
		24h	15.2	6.5	2.2	6.8
No. 4 2.3kg ♂	毎回 2ml	対照	10.1	5.1	1.9	6.2
		20m	10.8	5.1	2.1	6.4
		1h	10.5	4.9	2.0	6.4
		4h	9.9	5.0	2.3	6.4
		24h	10.2	5.2	2.2	6.1
No. 5 3.1kg ♂	毎回 5ml	対照	13.5	5.8	2.1	6.1
		20m	13.3	5.8	2.0	6.1
		1h	12.1	5.1	2.5	6.5
		2h	11.9	4.5	2.6	7.0
		24h	10.8	4.9	2.7	7.0
No. 6 3.0kg ♂	毎回 5ml	対照	11.1	5.4	1.5	5.9
		20m	10.9	5.0	1.6	6.1
		1h	9.9	4.9	1.9	6.5
		2h	9.9	5.1	2.2	6.7
		24h	10.8	4.8	2.2	6.9

表中の数字は mg/dl 示す。

つとした。

実験成績

a：弗化ナトリウム静注：10mg/kg 以下の量では，わずかに血清 Ca の減少を示すものもあつたが，有意の差はみられず，その他にはほとんど変化がみられなかつた。30mg/kg i.v. では血清Caおよび血清 Ca^{++} は明らかに減少した（第2表，第31図）。第2表の各実験例の血清 Ca の項において，対照値と各時間値とのそれぞれの差を求めて，これを平均すると 20^{m} 値では −1.7mg/dl，1^{h} 値では −3.0mg/dl，4^{h} 値では −2.8mg/dl であり，このうち 1^{h} 値および 4^{h} 値におけるこの差は最大であつて，いずれも有意であつた。血清 Ca^{++} の項においても，これと平行して減少し，1^{h} 値の対照との差は平均 −1.3mg/dl，4^{h} 値では −1.4mg/dl を示し，この差は有意であつた。いつぽう血清 Mg および血清 P はこれとは逆に上昇の傾向がみられるが，比較的変動が小で，上記と同様に検定すると，血清 Mg の 1^{h} 値と対照との差は平均 +1.0 mg/dl で有意であつたが，4^{h} 値における対照との差は平均 +0.7mg/dl で，この差は有意でなく，また血清 P の 1^{h} 値および 4^{h} 値における対照との差はそれぞれ +0.8mg/dl および +0.2mg/dl で，この差は有意でなかつた（第31図）。

第2表　弗化ナトリウム 30mg/kg i.v. による血清電解質の変動

ウサギ No.	時間	総Ca	透析性Ca	総Mg	無機P
No. 23 2.6kg ♂	対照	12.6	5.9	1.8	6.3
	20m	10.2	5.2	2.8	7.2
	1h	9.6	4.4	3.4	7.2
	4h	9.2	4.4	4.0	7.0
	24h	—	—	—	—
No. 24 2.8kg ♂	対照	13.2	5.4	1.5	5.6
	20m	13.2	5.2	1.9	6.2
	1h	10.8	4.3	1.8	8.6
	4h	10.2	4.3	1.6	6.2
	24h	13.3	5.2	1.4	6.0
No. 25 2.85kg ♂	対照	10.8	4.0	2.1	4.7
	20m	9.3	3.7	2.9	4.9
	1h	8.2	3.3	3.5	5.2
	4h	9.0	3.4	2.8	4.3
	24h	8.7	4.2	2.9	6.9
No. 31 2.44kg ♂	対照	12.4	5.2	1.3	6.1
	20m	10.0	4.0	2.1	7.4
	1h	7.6	2.9	2.3	6.1
	4h	9.0	3.6	1.6	5.0
	24h	—	—	—	—
No. 37 2.5kg ♂	対照	12.0	5.8	1.4	6.6
	20m	9.8	4.9	2.5	6.4
	1h	9.9	4.9	2.0	6.4
	4h	9.7	4.1	1.6	7.4
	24h	10.9	5.7	1.1	7.1
平均値	対照	12.2±1.2	5.3±1.1	1.6±0.5	5.9±1.0
	20m	10.5±2.1	4.6±1.0	2.4±0.6	6.4±1.4
	1h	9.2±1.8	4.0±1.2	2.6±1.1	6.6±1.8
	4h	9.4±0.7	3.9±0.6	2.3±3.0	6.0±1.8
	24h	11.0±7.0	5.0±2.3	1.8±2.9	6.7±1.8

表中の数字は mg/dl を示す。
(−) は実験を欠く。(危険率：5%)

第3表　クエン酸ナトリウム 72mg/kg i.v.による血清電解質の変動

ウサギ No.	時間	総Ca	透析性Ca	総Mg	無機P
No. 21	対照	15.2	8.8	0.8	—
2.6kg	20m	14.2	5.6	1.0	—
♂	1h	14.4	7.0	0.9	—
	4h	11.0	5.2	1.2	—
	24h	—	—	—	—
No. 22	対照	14.6	5.7	2.3	6.0
2.2kg	20m	13.0	5.5	2.8	6.4
♂	1h	12.0	5.8	2.7	7.2
	4h	14.8	5.9	2.1	7.5
	24h	—	—	—	—
No. 26	対照	10.6	5.1	1.9	5.9
2.9kg	20m	10.4	5.2	1.9	5.6
♂	1h	9.8	5.1	2.2	5.7
	4h	7.6	3.7	3.1	6.1
	24h	10.9	5.2	2.3	5.8
No. 27	対照	11.0	5.2	2.1	6.1
2.88kg	20m	10.4	4.3	1.9	5.0
♂	1h	10.6	4.7	1.9	5.0
	4h	11.2	4.3	2.2	4.6
	24h	10.9	4.8	2.3	4.0
No. 28	対照	12.0	5.0	1.9	5.8
3.0kg	20m	11.4	4.9	1.9	4.2
♂	1h	11.4	5.0	2.0	4.2
	4h	12.2	5.3	1.8	4.2
	24h	—	—	—	—
No. 29	対照	12.0	5.5	0.8	5.9
2.96kg	20m	12.0	5.3	1.8	6.9
♂	1h	12.0	5.0	0.8	6.9
	4h	10.0	4.7	1.9	7.1
	24h	11.0	5.1	2.1	6.8
平均値	対照	12.6±2.2	5.9±1.7	1.6±0.8	5.9±0.2
	20m	11.9±1.7	5.1±0.6	1.9±0.6	5.6±1.5
	1h	11.7±1.8	5.4±1.0	1.8±0.9	5.8±1.7
	4h	11.1±2.7	4.9±0.9	2.1±0.7	5.9±2.0
	24h	10.9±0.2	5.0±0.7	2.2±1.4	5.5±4.3

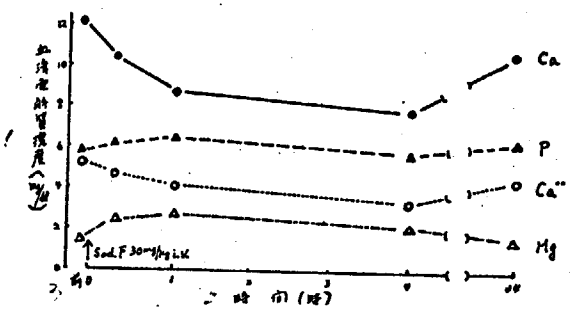
表中の数字は mg/dl を示す。
(－) は実験を欠く。(危険率：5%)

b. クエン酸ナトリウム静注：24mg/dl 以下の量では著変なく，72mg/kg による成績は第3表，第32図に示した。すなわち血清 Ca は時間とともに減少し，1～4h 値が最も低く，1h 値の対照との差は平均 －0.9mg/dl であり，有意であつた。4h 値の対照との差は平均 －1.1mg/dl であつたが，分散が大で有意の差はえられなかつた。血清 Ca^{++} は血清 Ca とほぼ同様の傾向を示したが，比較的恒常性を保ち，各時間毎の対照との差は有意でなかつた。血清 Mg は血清 Ca とは逆に軽度の増加傾向がみられたが，有意でなく，血清 P も変動が小で有意な差はえられなかつた（第3表，第32図）

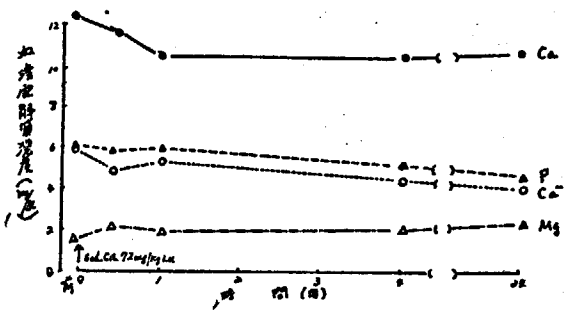
第4表　蓚酸ナトリウム 48mg/kg i.v.による血清電解質の変動

ウサギ No.	時間	総Ca	透析性Ca	総Mg	無機P
No. 42	対照	13.4	5.3	2.0	5.9
3.5kg	20m	7.0	2.7	1.8	5.9
♂	1h	7.4	3.4	2.2	5.0
	4h	8.6	4.3	4.0	4.1
	24h	—	—	—	—
No. 43	対照	10.9	4.3	1.8	3.4
2.84kg	20m	7.3	2.5	1.5	3.4
♂	1h	7.2	2.6	1.2	3.5
	4h	9.2	3.7	1.6	4.2
	24h	9.4	3.9	1.4	6.4
No. 44	対照	9.0	4.2	—	—
2.86kg	20m	6.5	2.8	—	—
♂	1h	6.5	2.9	—	—
	4h	6.5	2.9	—	—
	24h	7.1	2.7	—	—
No. 45	対照	13.8	5.3	1.0	—
3.31kg	20m	12.9	5.0	—	—
♂	1h	11.6	4.4	1.4	—
	4h	14.0	5.5	1.0	—
	24h	—	—	—	—
No. 46	対照	13.6	5.7	0.9	—
3.04kg	20m	9.0	3.4	1.2	—
♂	1h	10.4	4.2	1.4	—
	4h	12.2	4.8	0.9	—
	24h	—	—	—	—
No. 47	対照	9.2	4.2	1.2	4.1
2.88kg	20m	8.0	3.0	2.2	5.0
♂	1h	8.0	3.0	2.8	4.9
	4h	8.2	2.7	2.4	4.5
	24h	8.2	3.4	2.0	4.9
No. 48	対照	10.0	4.6	1.6	5.6
2.0kg	20m	8.4	4.1	1.2	5.6
♂	1h	6.4	2.7	2.8	5.4
	4h	9.6	2.2	2.6	5.7
	24h	11.0	4.9	1.4	5.5
平均値	対照	11.4±2.1	4.8±0.6	1.4±0.5	4.7±2.2
	20m	8.4±2.1	3.4±0.9	1.6±0.6	5.0±2.0
	1h	8.2±2.0	3.3±0.7	1.9±0.8	4.7±1.5
	4h	9.8±2.6	3.7±1.4	2.1±1.5	4.6±1.4
	24h	8.9±2.7	3.7±1.7	1.6±1.1	5.6±2.3

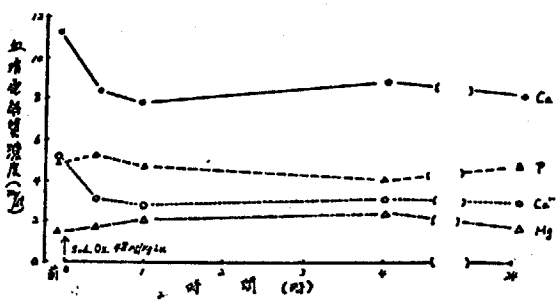
表中の数字は mg/dl を示す。
(－) は実験を欠く。(危険率：5%)



第31図　血清総カルシウム，透析性カルシウム，総マグネシウム及び無機燐の変動に及ぼす弗化ナトリウムの影響
（ウサギ5例平均）



第32図　血清総カルシウム，透析性カルシウム，総マグネシウム及び無機燐の変動に及ぼすクエン酸ナトリウムの影響
（ウサギ6例平均）



第33図　血清総カルシウム，透析性カルシウム，総マグネシウム及び無機燐の変動に及ぼす蓚酸ナトリウムの影響
（ウサギ7例平均）

c. 蓚酸ナトリウム静注：16mg/kg i.v.以下の量では軽度の血清Ca減少傾向を示すほかは著変なく，48mg/kg i.v.による成績は第4表，第33図に示した。すなわち血清 Ca および血清 Ca^{++} はすでに20m後から著明に下降し，1h 値が最も低く，対照との差は血清 Ca で －3.2mg/dl，血清 Ca^{++} で 1.5mg/dl であり，4h 値においてもなお対照との差は血清 Ca で －1.7mg/dl，血清 Ca^{++} で －1.1 mg/dl を示し，これらの差はいずれも有意であつた。血清 Mg はこれらと逆にやや増加の傾向を示すが，No.43例のように軽度減少するものもあり，変動も小で有意な差はえられなかつた。また血清 P の変動も比較的小で，しかも分散が大きく，確かな成績はえられなかつた（第4表，第33図）。

Ⅱ　ラットの呼吸ガス代謝ならびに体温に及ぼす作用

実験材料ならびに方法

実験動物として体重 200g 前後の雄性成熟ラットをえらび，一定期間飼育したのち実験に使用した。実験装置は宮田・赤羽の Knipping 型呼吸ガス代謝測定装置小動物用改良型を使用した。同装置の構造及び使用法は既報の両氏[20][21]報告に従い，また実験にさいしての注意事項もこれに準じた。動物は実験前によく装置に馴らすようにし，ついで，10m間の O_2 消費量及び CO_2 排出量を測定した。CO_2 吸収には 12% KOH 15ml，CO_2 駆出には 20% H_2SO_4 10ml を

用いた。

CO_2/O_2 を呼吸商とし，基礎代謝量は Knipping 法によつて算出した。かくしてえた呼吸ガス代謝量及び基礎代謝量は，それぞれ毎 kg 毎分量及び毎 kg/24h 量として記載した。基礎代謝値は実験当日に反覆測定して最も安定したと認められた値をえらび，これを注射前の対照値とした。動物は実験前15～20h 絶食せしめ，全実験を通じて室温20～22°C，動物室温は22°C 前後に保つた。

体温の測定には無留点屈曲体温計を使用し，呼吸ガス代謝測定後ただちに直腸内 5cm の深さにて 3min 間以上測定した。

使用薬物は予め生理食塩水溶液とし，注射全量が1mlを越えないようにして腹腔内に投与した。以下記載の実験成績は3例平均値である。

実験成績

a. 弗化ナトリウム：5mg/kg 腹腔内注射では，呼吸ガス代謝及び体温に著変なく，20mg/kg では注射後第1h の O_2 消費量が 18.7ml/min/kg（対照値 22.6ml/min/kg）で17%の減少，以後回復して第3h で前値にもどり，CO_2 排出量は注射後第 1h の測定値が 16.4ml/min/kg（対照値 17.2ml/min/kg）で 5% の減少，以後回復に向ひ第 2h，第3h ともに 18.7ml/min/kg で 9% の増加を示した。呼吸商は上昇し，体温は第 1h 後 35.5°C で 18°C の下降を示したが，そのご回復して第3h で正常に復した。30mg/kg 腹腔内注射では，O_2 消費量は注射後第 1h の測定値が 20.2ml/min/kg（対照値 27.0 ml/min/kg）で25%の減少を示し，以後徐々に回復するが，第 2h 値は 23.9ml/min/kg でなお12% の減少がみられた。しかし第 3h ではほとんど前値に復した。

CO_2 排出量は注射後第 1h の測定値が 17.2ml/min/kg（対照値 23.8ml/min/kg）で28%の減少，以後回復に向い第2hは 20.1ml/min/kg で16%の減少，第 3h ではほとんど前値に回復した。呼吸商は下降の傾向がみられた。体温は第 1h 後35.9°C で，2.6°C の著明な下降を示し以後回復に向うが，第 3h に至るもなお 0.8°C の下降を示した（第5表，第34図）。

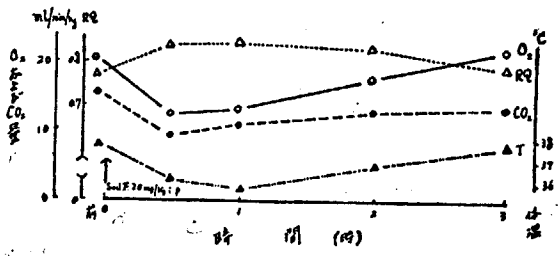
b. クエン酸ナトリウム：48mg/kg 腹腔内投与では O_2 消費量は注射後第 1h の測定値が 31.3ml/min/kg（対照値 26.6ml/min/kg）で18%の増加，以後急速に回復に向い，第 2h ではほとんど前値に復した。CO_2 排出量は注射後第 1h の測定値が 26.6ml/min/kg（対照値 23.5ml/min/kg）で13%の増加をみたがただちに回復し，第 2h 値はほとんど前値に復した。呼吸商は著変なく，体温もほとんど変動がみられなかつた（第6表，第35図）。

c. 酒酸ナトリウム：8mg/kg の腹腔内投与ではほとんど著変なく，32mg/kg では O_2 消費量は注射後第 1h の測定値が 23.2ml/min/kg（対照値 25.0ml/min/kg）で7%減少，そのごさらに減少を示し，第2h 値21.4ml/min/kgで14%減少を示し，以後徐々に回復に向うが，第3h値23.2ml/min/kgでなお7%の減少を示した。CO_2排出量は注射後第1hの測定値が 19.7ml/min/kg（対照値 21.5ml/min/kg）で 8%，第 2h 値 18.0ml/min/kg で 16%の減少を示し，以後回復に向うが，第 3h 値 18.9ml/min/kg でなお12%の減少を示した。呼吸商は下降の傾向，体温は第 1h 後 37.0°C で 0.8°C の下降を示したが，まもなく回復して第2h ではほとんど前値に復した（第7表，第36図）。

第 5 表　ラット呼吸ガス代謝及び体温にたいする Sod. F., Sod. Cit., Sod. Ox. の作用

	時間	体温 °C	O_2 消費量 ml/min/kg	CO_2排出量 ml/min/kg	呼吸商	基礎代謝 Cal/24h/kg
弗化ナトリウム 30mg/kg i.p.	対照	38.5	27.0	23.8	0.89	192
	1h	35.9	20.2	17.2	0.86	144
	2h	37.0	23.9	20.1	0.85	169
	3h	37.7	25.4	22.4	0.88	182
弗化ナトリウム 20mg/kg i.p.	対照	37.3	22.6	17.2	0.76	157
	1h	35.5	18.7	16.4	0.88	133
	2h	36.5	20.3	18.7	0.92	145
	3h	37.5	33.5	18.7	0.80	164
クエン酸ナトリウム 48mg/kg i.p.	対照	38.1	26.6	23.5	0.88	189
	1h	38.1	31.3	26.6	0.85	222
	2h	38.0	25.8	23.5	0.90	185
	3h	38.0	26.6	23.5	0.88	189
酒酸ナトリウム 32mg/kg i.p.	対照	37.8	25.0	21.5	0.86	178
	1h	37.0	23.2	19.7	0.85	165
	2h	37.5	21.4	18.0	0.84	152
	3h	37.9	23.2	18.9	0.82	164

3 例平均値



第34図　ラット呼吸ガス代謝並びに体温に及ぼす弗化ナトリウムの影響
ラット 205g，♂，NaF：20mg/kg i.p.

X　弗化ナトリウムの血液 Catalase ならびに肝 Xanthine-Oxidase におよぼす作用

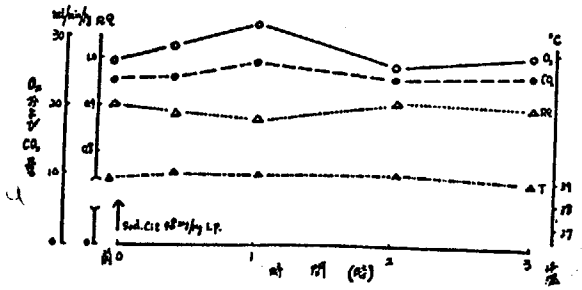
実験材料および方法

1. Catalase：ウサギ血液の H_2O_2 分解能を Warburg 法により測定した，弗化ナトリウムは $M/_{300}$ となるようにして，酵素材料（採血直後のウサギ全血を冷純水で 100 倍稀釈）と混じてもちいた。恒温槽温度は 20°C とし，振盪回数は 80/min とし，測定は 4min 間隔で30～50min 間にわたつておこなつた。

第 6 表　弗化ナトリウムの血液 Catalase にたいする作用

実験番号	反応	測定時間（分） 2	6	10	14	18	22	26	30	35	40
1	対照	69.0	134.0	188.0	231.0	266.0	296.0	317.0	334.0	350.0	361.0
	NaF	11.2	24.3	37.0	48.9	61.0	71.0	81.5	91.0	101.0	110.0
2	対照	38.2	93.0	131.0	156.0	198.0	223.0	245.0	264.0	286.0	304.0
	NaF	6.85	13.8	20.0	25.8	32.5	38.0	43.8	48.0	58.0	62.1
3	対照	15.9	36.8	53.7	65.5	76.5	84.5	95.5	102.0	108.0	112.0
	NaF	3.95	10.2	19.0	22.9	28.8	34.0	40.4	45.6	50.5	57.0
4	対照	41.2	92.0	131.0	167.0	198.0	226.0	249.0	270.0	294.0	309.0
	NaF	4.05	10.5	17.6	23.6	29.8	35.6	40.8	46.5	53.0	58.5
5	対照	63.4	125.0	177.0	220.0	254.0	277.0	300.0	313.0	327.0	334.0
	NaF	7.0	16.6	30.3	43.6	57.0	65.4	79.5	88.6	99.5	113.0
5例平均	対照	45.7	95.2	136.1	167.9	198.4	211.3	241.3	256.6	273.0	284.0
	NaF	6.6	15.1	24.8	33.0	41.8	48.8	57.2	63.9	72.4	80.1

数値は O_2-Evolution（μl）を示す。



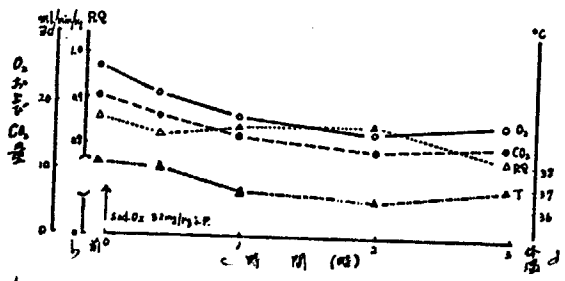
第35図　ラット呼吸ガス代謝並びに体温に及ぼすクエン酸ナトリウムの影響
ラット 210g，♂，Sod. Cit：48mg/kg i.p.

2. Xanthine-Oxidase：酵素材料にはラット肝ホモジネート（冷純水 1cc 中 160mg の組織含有）を，基質には Xanthine をもちい，Axelrod-Elvehjem[21]の方法に従つて Warburg 装置にて測定をおこなつた。各実験において基質たる Xanthine の存在しないときの O_2 吸収（Endogenous respiration）を測定し，真の Xanthine 酸化速度を求めるさいの補正をおこなつた。すなわち第38図にしめした[illegible]な4つの反応系をつくり，（1）ホモジネートの

第7表　弗化ナトリウムの Xanthine Oxidase ならびに Endogenous Respiration にたいする作用

実験番号	測定反応系	測定時間(分) 20	40	60	80	100	120	140	160	180
1	E.R.	29.8	48.8	59.5	65.5	76.1	89.3	98.0	101.0	115.0
	E.R.+NaF	22.0	34.6	47.5	53.4	56.7	60.3	61.8	61.7	63.0
	E.R.+Xanth.	43.7	64.2	73.5	85.0	95.5	100.0	121.1	123.0	138.0
	E.R.+Xanth.+NaF	29.6	40.2	49.3	53.8	57.3	60.4	63.4	65.3	66.1
2	E.R.	29.3	47.7	55.4	62.4	63.9	74.0	83.0	94.6	112.0
	E.R.+NaF	35.8	50.2	59.3	65.7	69.0	71.8	74.5	79.2	82.6
	E.R.+Xanth.	45.7	63.9	70.0	78.5	84.0	90.1	100.0	111.0	123.0
	E.R.+Xanth.+NaF	32.0	45.0	51.9	58.9	62.3	64.5	68.1	70.7	71.6
3	E.R.	27.0	39.2	48.5	57.0	61.0	67.6	73.0	81.5	88.5
	E.R.+NaF	20.9	28.7	34.0	42.1	45.6	49.8	50.6	52.3	54.1
	E.R.+Xanth.	28.4	39.6	44.3	53.4	57.0	61.0	66.5	75.0	82.5
	E.R.+Xanth.+NaF	27.4	33.6	39.8	44.3	47.8	49.9	53.0	55.6	57.8
平均	E.R.	28.7	45.2	54.5	61.6	67.0	77.0	84.7	92.4	105.2
	E.R.+NaF	26.2	37.8	46.9	47.0	57.1	60.6	62.3	64.4	66.6
	E.R.+Xanth	39.0	55.9	62.6	72.3	78.8	83.7	95.8	103.0	114.5
	E.R.+Xanth.+NaF	29.7	39.6	47.0	52.3	55.8	58.3	61.5	63.9	65.2

数値は O_2 吸収量（μl）を示す。



第36図　ラット呼吸ガス代謝並びに体温に及ぼす蓚酸ナトリウムの影響
ラット 220g, ♂, Sod. Ox.: 32mg/kg i.p.

Endogenous respiration (E.R.) を，(2) で E.R.にたいする弗化ナトリウムの影響を，(3) では (1) に Xanthine を添加して E.R.による O_2 吸収と Xanthine 酸化による O_2 吸収をあわせ測定し，(4) では (3) にたいする弗化ナトリウムの作用を観察した。

実験成績

1. Catalase activity にたいする弗化ナトリウムの作用

成績は第37図にしめすごとく，すべての実験例において，Catalase による H_2O_2 分解能は弗化ナトリウムの添加によつて著明に低下し，30m 後の O_2 発生率は対照に比して約 1/4 となる（第37図）。

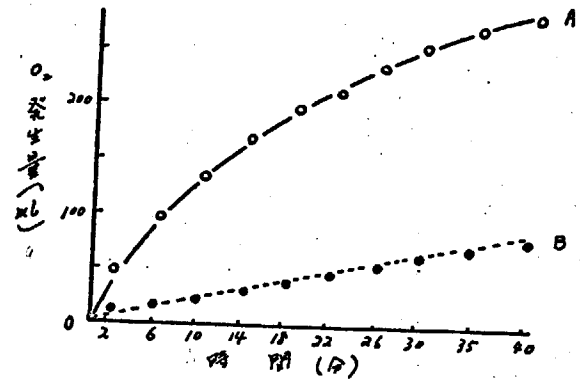
2. Xanthine-Oxidase activity にたいする弗化ナトリウムの作用

成績は第38図にしめすごとく弗化ナトリウムは Xanthine 酸化による O_2 摂取にたいしては一定の影響をあたえなかつた。

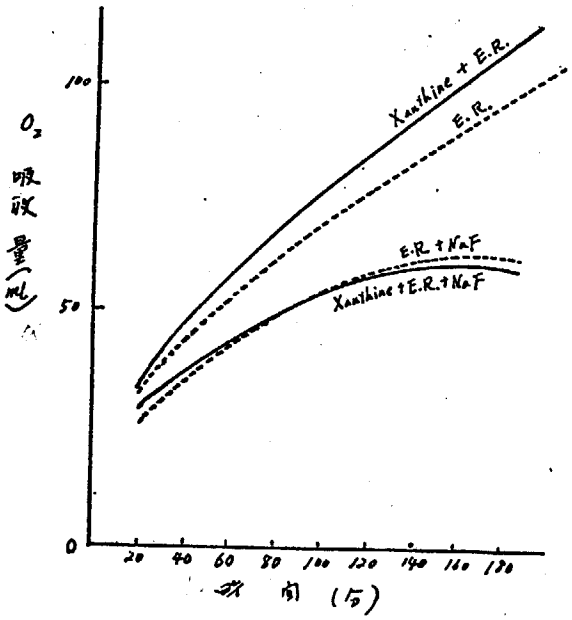
なおこの実験においては肝ホモジネート自身の E.R.による O_2 摂取がかなり大で Xanthine 酸化による O_2 摂取との差がすくなく，成績の判定は困難であつたが，Xanthine-Oxidase activity には明らかな抑制作用はみられなかつたが，Endogenous respiration (E.R.) にはすべての例で抑制傾向がみとめられた。

考　察

生体内における急性弗素中毒症状，あるいは摘出臓器組織にたいする弗素の作用が，脱カルシウム作用の結果であるとする学者 (Schultz, Schwyzer, Loewi その他) と主として酵素阻害作用あるいはそれ以外の弗素イオンじしんの特異な作用によると考えている学



第37図　弗化ナトリウ血液 Catalase にたいする作用
A：対　照
B：弗化ナトリウム



第38図　弗化ナトリウムの Xanthine Oxidase ならびに Endogenous Respiration (E. R.) にたいする作用

者 (Sollmann, Bannet, Covin & Berman, その他) とのあいだに意見の対立のあることはすでに緒言においても述べた通りである。著者はこれらの疑問の一端を解明する一手段として，弗素と同様にカルシウムと強い結合性をもつクエン酸ナトリウムおよび蓚酸ナトリウムをもちいて，これら薬物が in vitro で同一量のカルシウムと結合する濃度または量（すなわち同一 Equivalent concentration）においてその薬理作用を比較実験した。

クエン酸ナトリウムの毒性は比較的小で，ふるくから血液凝固抑制剤として，主として輸血，赤沈検査などの際にもちいられているが，近来外科手術の発達にともなつて大量輸血が行われるにいたつて，クエン酸中毒例が報告されるようになつた。John ら[83]はクエン酸加血輸血のさいみられた急性クエン酸中毒患者の幾多の臨床例を綜合して検討し，これらはいずれも低カルシウム血症を伴うが，必ずしもカルシウム静注によつて救われなかつたこと，および肝疾患々者に急速かつ頻回のクエン酸加血液を輸血した場合はきわめてその中毒を起しやすいことなどを報告している。クエン酸ナトリウムの致死量は，池田の致死量集[8]にはウサギ静注 MLD 0.4～1.6g イヌ静注 MLD 0.37g と記載されている。

蓚酸ナトリウムの毒性はかなり大で，Sollmann および Goodman & Gilman の各教科書によれば，これが多量に体内に吸収されると強い局所作用のために痙攣が誘発し，嘔吐，腹痛，虚脱およびけいれんをおこし，低カルシウム血症による種々の症状のほか，難溶性のカルシウム塩が細尿管を閉塞して仙痛，乏尿，無尿，血尿あるいは腎炎などを起すという。池田の動物致死量集によれば，蓚酸ナトリウムのウサギ皮下注射 MLD は 0.25g，モルモット皮下注射 MLD 0.4g/kg.静注 0.1～0.2g/kg と記載されている。

本実験成績からマウスにたいする急性中毒症状および LD_{50} を比較すると，弗[illegible]リウム 100mg/kg

i.p.では注射後まもなく激しい興奮症状がみられ，けいれんにつづく麻痺が現れて，30～40^{m}で呼吸停止して死亡した。弗化ナトリウムの LD_{50} は 71.5～75.8 mg/kg（危険率：5%）であつた。淵川[42]は弗化ナトリウムを水溶液として，雌雄のマウスに腹腔内注射して，ほぼ著者と同様の中毒症状を観察し，LD_{50} は約 50mg/kg と算定しているが，著者はウサギの実験のさい 50mg/kg i.v. しても死亡例は少なく，腹腔内投与では3例中死亡したものはなかつた。

弗化ナトリウム 100mg/kg と同一 Equivalent concentration のクエン酸ナトリウム（240mg/kg）ではわずかに呼吸興奮が軽度にみられたにすぎず，10倍量の 2.4g/kg i.p.では直後から著明な呼吸促迫，同不整，立毛，拳尾反応および反弓伸張などの強い中枢興奮症状がみられ，ついで強直性けいれんが現れ，のち麻痺に移行して30～40^{m}前後で死亡するものもあるが，なお数回のけいれん発作にたえて生存する例が多くみられた。クエン酸ナトリウムの LD_{50} は 2.67～3.09g/kg（危険率：5%）であつた。

蓚酸ナトリウム（160mg/kg）i.p.では軽度の運動量減少と鎮静状態がみられるにすぎず，5倍量 800 mg/kg のでは呼吸不整につづく立毛などがみられたが，反射性亢進などの症状は著明でなく，むしろ不穏状から鎮静麻痺に移行して30^{m}前後で強直性けいれん，揺搖などがあらわれ呼吸停止して死亡した。蓚酸ナトリウムの LD_{50} は 345～385mg/kg（危険率：5%）であつた。

すなわちマウスにたいする毒性は弗化ナトリウムが最も強く，蓚酸ナトリウムはその約 $^{1}/_{5}$ 前後で，クエン酸ナトリウムは弗化ナトリウムの $^{1}/_{40}$ 以下であり，また急性中毒時著明な中枢興奮症状のみられたのはクエン酸ナトリウムであり，死亡例はつねに反弓伸張，拳尾反応などの症状がみられたが，これと比較すると弗化ナトリウムはたとえ大量でもそれほど著明な症状は示さず，蓚酸ナトリウムは興奮症状よりも鎮静麻痺にはやく移行した。

クエン酸ナトリウムの中枢興奮症状はマウス LD_{50} からみては極めて少ない量（48mg/kg）をウサギに静注しても明らかにみられるが，かなり大量でも注射を極めてゆつくりと行なうときは興奮症状は比較的短時間で回復する。これは体内に入つたクエン酸の酸化速度に関係するためとおもわれる。

ラットにたいする呼吸ガス代謝の面から毒性を比較すると，弗化ナトリウム 5mg/kg，クエン酸ナトリウム 12mg/kg，蓚酸ナトリウム 8mg/kg i.p.では，いずれも呼吸ガス代謝ならびに体温には著変なく，弗化ナトリウム 20mg/kg，クエン酸ナトリウム 48 mg/kg，蓚酸ナトリウム 32mg/kg 腹腔内注射では3者とも特徴ある傾向を示した。すなわち弗化ナトリウムは O_2 消費量は第 1^{h} 値17%減少，CO_2 排出量は第 1^{h} 値 5% の減少を示したが，以後は回復に向い 3^{h} で全く正常に復した。体温は第 1^{h} 値 1.8°C と著明に下降したが，以後徐々に回復して，3^{h} では全く正常にもどつた。30mg/kg 注射ではさらにこれらの傾向は著明に現れ，かつ持続時間も長かつた。

これにたいし，クエン酸ナトリウムでは O_2 消費量は第 1^{h} 値18%上昇，CO_2 排出量は第 1^{h} 値13%の増加を示し，以後回復に向つて第 2^{h} 値はほとんど正常に回復した。体温にはこの量では全く影響を及ぼさなかつた。蓚酸ナトリウムでは O_2 消費量が第 1^{h} 値 7 %，CO_2 排出量は 8% の減少を示し，そのごさらに第 2^{h} 値では O_2 消費量は14%減少，CO_2 排出量は 16 %の減少を示し，以後回復に向うが，第 3^{h} 値でもなお前値以下であつた。体温は第 1^{h} 値 0.8°C 下降したが以後回復に向つて第 2^{h} にはほぼ正常に回復した。

Goodman & Gilman の教科書によれば，弗素イオンは酵素活動を阻害し，摘出臓器の Anaerobic glycolysis を低下せしめ，組織呼吸を抑制し，筋肉の酸素消費と乳酸産生を抑制せしめるが，これと同様に生体内でも O_2 消費を減少せしめ，CO_2 産生を減少させるという。著者の成績からもこのことはいいうることで，弗化ナトリウム 20mg/kg 腹腔内投与ラットの呼吸ガス代謝を抑制せしめた。このさい体温下降が毎常みられたが，O_2 消費量の減少にともなつて下降し，回復もほぼ同時間であるところから，弗素イオンの毒性による代謝機能低下の結果熱生産が減退したためであろう。

クエン酸ナトリウムはどの動物についても3薬物中最も中枢興奮症状が激しく，とくに呼吸興奮は比較的小量でも著明にみられた。本実験において，クエン酸ナトリウムが，マウス LD_{50} に比較してはるかに小量の 48mg/kg i.p.で，ラットの O_2 消費量ならびに CO_2 排出量が第 1^{h} 値に増加を示したのは一般代謝機能が一過性に亢進し，クエン酸の体内酸化が迅速に行われるためであろう。

蓚酸ナトリウムの O_2 消費量，CO_2 排出量の減少が第 2^{h} 値において著明であり，持続時間も長かつたが，このような時間的傾向は他の実験においてもしばしばみられたところで，この薬物が体内に吸収されてからは難溶性のカルシウム塩となるため，以後の分解排泄が遅れるためか，あるいは蓚酸じしんの組織毒のために代謝機能の減退が徐々に現れかつ長時間にわたつて続くのであろう。体温下降が O_2 消費量に並行して下降する点は，弗化ナトリウムの場合と同様にやはり代謝機能の減退もしくは抑制による熱生産低下によるものとおもわれ，このものの毒性は決して軽視できない。

急性弗素中毒症状が血清カルシウム（以下血清 Ca と略記）減少に起因するかどうかは別問題として，大量投与時に血清 Ca が減少することはすでに多くの学者によつて一般に認められているところである。しかしこれにともなう他の血清電解質については意見はまちまちである。たとえば血清マグネシウム（以下血清 Mg と略記）についての報告はあまりみあたらず，血清無機燐（以下血-Pと略記）は Marcovitch ([illegible]による）は減少するといい須川[illegible]，Bogdanovic[illegible]らは増量するといい確かではない。

クエン酸ナトリウム及び蓚酸ナトリウムについては，Tsai & Hsu[illegible]はイヌに各 50mg/kg 静注して，血清 Ca 及び血清 P の著明な減少を認め，5～7^{h} で最低となり，24^{h} 後にはほとんど回復したと報告している。石見らはさいきん弗化ナトリウム，クエン酸ナトリウム及び蓚酸ナトリウムをネコに各 50mg/kg 静注し，血圧実験と並行して血清中のカルシウムの変動を検べ，いずれも著明血清 Ca の減少をみたことを報告している。Bunker ら[illegible]はクエン酸加血液の大量輸血を行つた130名の患者について，血清 Ca，血清 Ca^{++} の著明な減少をみたが，このさい血清 Mg および血清 P 量に有意の差はなかつたと報告している。

しかしこれまでの多くの研究者らの行つた血清 Ca の測定は，定量法の関係からかなり大量の血清を必要とし，従つて1回の採血量が多量になるため，ときどき採血して時間的経過を追つてその変動をしることは困難である。著者は柳沢法[illegible]によつて血清 Ca 量の測定を行つたが，1回の採血量を 5ml とし，時間毎に数回にわたつて採血すれば，全く薬物の影響なしに血清 Ca 量は著明に減少し，血清 P は逆に増量することを知つた。柳沢法は試料小量で可能という特長があるので，1回2ml ていどの採血で時間毎に数回の採血をしても，貧血による影響はほとんどないことがわかつた。そこで柳沢法によつて，これら3薬物のウサギ血清 Ca，血清 Ca^{++} および血清 Mg の変動をしらべ，同時に Fiske & Subbarow 法によつて血清 P の変動をも研究した。採血時間はウサギ血圧実験を参考とし，薬物投与前を対照に，薬物静注後血圧最低となる 15～20^{m}，血圧がほとんど回復する 1^{h}，4^{h} および2 4^{h} とした。

弗化ナトリウム 30mg/kg，クエン酸ナトリウム 72 mg/kg，蓚酸ナトリウム 48mg/kg でこれを比較すると，血清 Ca 及び血清 Ca^{++} は弗化ナトリウムでは注射後1～2^{h} が最も低く，以後回復にむかうが，なかには 4^{h} 後においてもなおかなり低い値を示すものもあつた。24^{h} 後にはほとんど正常に回復した（第2表，第31図）。クエン酸ナトリウムでは比較的減少度が小で，20～60^{m} が最低を示し，以後回復にむかい 4^{h} にはほとんど対照との差はなかつた（第3表，第31図）。蓚酸ナトリウムはほぼ弗化ナトリウムと同様の傾向を示したが，Ca 減少度は最も著しかつた。

血清 Mg は弗化ナトリウムでは時間とともに軽度の増加を示し，Ca と逆相関を示したが，他の2者は確かな成績がえられなかつた。

血清 P は弗化ナトリウムでは注射後20～60^{m} 値に増加を示すものが多く，以後回復に向つたが有意の差はえられなかつた。クエン酸ナトリウムではほとんど変化なく，蓚酸ナトリウムは一般に時間とともに増加の傾向を示したが，いずれも変動が小さく有意の差はえられなかつた。

弗化ナトリウムの心臓作用については古くから多くの研究がある。Vernon[illegible]はカメの心臓を，De Nitto[illegible]は摘出哺乳動物の心臓を，Chang[illegible]はウサギの心房標本をもちいてその抑制作用のみを認めた。しかし Gottdenker u. Rothberger[illegible]はカエルの心臓並びにイヌの心肺標本をもちいて抑制作用を認める一方，モルモットやウサギの心房では，0.003M の濃度では逆に振幅の増大作用と同時に洞－房，房－室間の伝導障害と著明な不整脈があらわれることを指摘した。さらに Ferrannini[illegible]は 0.0024～0.0072M の濃度ではウサギの心房条片の収縮力増強を認め，Webb[illegible]は心筋の機能と種々の代謝物質との関係をしらべ，弗化ナトリウムが解糖作用を完全に阻止する濃度すなわち 0.005M～0.05M の濃度では，ウサギ心房標本にたいして最初振幅並びに搏動数を抑制するが，そののち搏動数は一定し，振幅はかえつて増大の傾向を示すと報告した。

いつぽう Loewi[illegible]は摘出カエル心臓にたいして，弗化ナトリウムが低濃度（0.02%）では Positive inotropic action があることを認め，その作用機序を弗素が心筋の Ca^{++} を奪つて不溶性の CaF_2 となり，心筋の細胞膜に沈着し，このものが心筋に作用するためと考えた。しかし Bennet[illegible]らは同様の効果について，弗素と心筋の銅イオンとの反応によるものと説明し，さらに Covin & Berman[illegible]らはラットの心筋標本にみられた弗化ナトリウムの Posi[illegible] inotropic effect を酵素阻害作用の面から考察し[illegible] T の酵素

酸化過程が阻害され，燐酸結合の高エネルギーが心筋収縮に利用されるであろうと述べている。さいきん瀬川はウサギの心房標本および心室内乳頭筋標本をもちいて弗化ナトリウムの作用機序を解明しようとした。その結果，心房標本では弗化ナトリウムは一時的に振幅を抑制してもほとんど不整脈をきたさないこと，搏動数の減少は振幅回復後もなお続くことなどから，刺激伝達の抑制が主体をなすものと考えた。

このように弗素の心臓にたいする作用については抑制と興奮との全く相反した報告がなされ，したがってその作用機序についての見解もさまざまである。著者のカエルおよびヒキガエル心臓にたいする実験成績から，まず生体内カエル心にたいする作用を弗化ナトリウム（2×10^{-4}），クエン酸ナトリウム（4.8×10^{-4}），蓚酸ナトリウム（3.2×10^{-4}）の順に比較してみると，弗化ナトリウムでは軽度の振幅増大と搏動数の減少を，クエン酸ナトリウムではほとんど変化なく，蓚酸ナトリウムでは軽度の振幅減少，搏動数の増加がみられ，すでに抑制効果があらわれている。高濃度では弗化ナトリウムと蓚酸ナトリウムはいずれも著明な抑制がみられるが，クエン酸ナトリウムは緊張低下を示した程度であつた。

摘出カエルおよびヒキガエル心にたいしては，弗化ナトリウム（2×10^{-5}）では振幅の増減不定で，とくに Positive inotropic effect のみを認めるわけにはゆかなかつたが，クエン酸ナトリウム（4.8×10^{-5}）は著明な振幅増大を示し，蓚酸ナトリウム（3.2×10^{-5}）はほぼ弗化ナトリウムと同様の傾向を示した。しかしこれより高濃度になると，3者とも著明な抑制効果がみられた。著者はこの抑制効果が Ca^{++} 欠乏に由来するものか，あるいは Ca と結合した形としての作用であるかをみるために，弗化ナトリウム（2×10^{-4}），クエン酸ナトリウム（4.8×10^{-4}），蓚酸ナトリウム（3.2×10^{-4}）の各溶液に脱 Ca 相当量の $CaCl_2$ を加え，薬物単独灌流時の作用と比較して興味ある所見をえた。すなわち摘出ヒキガエル心（八木式灌流法）にたいし，弗化ナトリウム（2×10^{-4}）単独灌流時では著明な振幅・搏動数の減少と伝導障害がみられるが，$CaCl_2$ 加灌流液では振幅の減少はみられず，かえつて軽度の増大傾向がみられた。しかし伝導障害はつねに現れた。さらに $CaCl_2$ を追加しても振幅のみが増大して伝導障害の改善はあらわれなかつた。

これにたいしクエン酸ナトリウム（4.8×10^{-4}）では著明な振幅抑制がみられるが，伝導障害は必ずしも全例にはみられない。$CaCl_2$ 加灌流液で灌流すると，直後一過性の振幅抑制はみられるがまもなく回復し，以後は Ringer 液灌流と全く異なるところがなかつた。蓚酸ナトリウム（3.2×10^{-4}）の心抑制効果は最も著明で，3.2×10^{-5} 溶液でもまれに振幅減少と伝導障害がみられることもあつた。しかし 3.2×10^{-4} 溶液に $CaCl_2$ を加えた灌流液では，直後一過性の振幅減少と緊張低下を示すがまもなく回復し，以後は全く正常の搏動をつづけた。Gottdenker & Rothberger は摘出カエル心臓を弗化ナトリウムと同濃度の弗化カルシウムで灌流せしめたとき，ナトリウム塩の場合より，やや遅れて同様の伝導障害がみられ，また哺乳動物の摘出心を Ringer 液中に浸した場合，Ca^{++} 量が正常の $^1/_{10}$ 程度になると心臓が弛緩状態で停止するのに反し大量の弗化ナトリウムは心臓を半収縮状態のまま停止することを実示し，これらの事実から弗化ナトリウムによる Ca^{++} 減少が，ただちに心臓抑制効果をもたらすものとは考えられないと結論づけた。著者の実験成績からも，クエン酸ナトリウムおよび蓚酸ナトリウムの場合とは明らかに異つて，Ca^{++} 追加の影響は弗化ナトリウムによつて現れる伝導障害になんの効果ももたらさなかつた。すなわち弗化ナトリウムの心臓抑制作用は，単に Ca^{++} 減少の結果ではなく，弗素イオンじしんのもつ特異な心臓作用が重要な意義をもつものであろうと推察される。瀬川もウサギ心房標本についてほぼ著者と同様の結論をえている。もちろんこれだけの実験事実では心臓作用機序を云々することは不能ではあるが，弗素イオンの特異な心臓作用を解明するための一つの手がかりになりうるとおもう。

神田ら[65]はウサギの耳介血管による血管中枢の新しい実験方法を発表したが，この方法によつて著者の実験成績を比較すると，弗化ナトリウム 30mg/kg，クエン酸ナトリウム 72mg/kg，蓚酸ナトリウム 48mg/kg 静注では，いずれも血管中枢の抑制作用がみとめられ，とくに弗化ナトリウムは著明であり，クエン酸ナトリウムは一過性軽度であり，蓚酸ナトリウムは比較的軽度ながら持続的（30^m 前後）であつた。しかし実験例数が少ないので薬物投与量とその拡張度との関係を確かめることはできなかつた。今後さらに詳細な研究を続けたいとおもう。

角田ら[66]は摘出ウサギ耳介ならびにヒキガエル後肢の血管灌流法によつて，弗化ナトリウムの末梢血管作用をしらべ，0.05%液灌流でいずれも著明な収縮をみたと報告しているが，著者の実験成績も全く一致して，血管中枢作用とは相反した末梢血管の収縮作用を確認した。しかし1%以上になると末梢血管はかえつて拡張し，2%では著明な滴数増加がみられた。しかもこのさい Ringer 液に交換すると，まもなく滴数減少し，完全に停止することもあつた。この作用が後述する弗化ナトリウムの血圧作用と関連しうるかどうかは別として，今回は単なる実験事実として報告するにとどめておく。これにたいしクエン酸ナトリウムおよび蓚酸ナトリウムは著明な末梢血管作用はみられず，しいていえばきわめて軽度の拡張がみられたにすぎない。蓚酸ナトリウム1.6%灌流時にみられた滴数減少は，薬物の作用でなく不溶性の蓚酸カルシウムによる器械的現象であるかもしれない。

急性弗素中毒時にみられる特徴の一つは著明な血圧下降であり，これは一般に一致して認めるところである。しかし，その作用機序に関しては意見の一致をみない。Schultz[6]，Schwyzer[9]，Greenwood et al[10]，McClure[11]および石見ら[12]は弗素の脱カルシウム作用による血管壁の緊張低下の結果であると結論づけている。Richardson ら[17]はイヌに少量（F として 5mg/kg）の弗素ナトリウム又は弗化錫を経口投与したさいでも，軽度ながら血圧下降があらわれ，この量では血清 Ca にはほとんど影響がないと報告し，さらに Goodman & Gilman の記載によると，弗素によつて起る降圧作用は心筋抑制と血管運動中枢抑制の結果であるという。

ウサギの血圧・呼吸・心動の実験からえられた著者の成績は，弗化ナトリウム 30mg/kg 静注で，血圧は注射後 15^m 前後で最低となり，約 20mmHg 下降したが，30^m ころにはほぼ回復した。心動は一過性に振幅抑制がみられたが，血圧下降が著明となるにつれて増大し，30^m 前後でほぼ回復した。注射量が増すとその作用はさらに著明に現れ，持続時間も長かつた。

これと比較して行つたクエン酸ナトリウム72mg/kg 静注では，血圧は一過性に著明に下降し，心動振幅も減少，緊張も著明に低下するが，注射後 5～10^m でほとんど回復した。このさい呼吸興奮も著明で，振幅は前値の4倍前後に増大し，そのご一時抑制されたが，30^m ころから再び増幅する例が多くみられた。蓚酸ナトリウム 48mg/kg i.v. では血圧は徐々に下降するが，回復時間も遅れ，持続時間は3薬物中最も長かつた。心動振幅は抑制され 30～40^m ごから徐々に回復を示した。

このようにいずれも血圧下降，呼吸興奮をみる点は類似しているが，その程度，作用時間あるいはそのごの経過などに3者3様の特徴がある。そこで血圧下降の作用機序を今回の著者の行つた心臓ならびに血管系にたいする実験成績と，血清 Ca の変動についての成績などから綜合して考察すると，まず弗化ナトリウムの場合には摘出カエル心の抑制効果（伝導障害）は Ca^{++} 添加によつてもこれを完全に除去することはできなかつた。いつぽう Gottdenker らはカエル，カメの心臓ならびにイヌの心肺標本について，弗化ナトリウムでみられた伝導障害が，これと同濃度の弗化カルシウムを作用させた場合にも同様の伝導障害をみたと報告している。著者の摘出ヒキガエル心にたいする Ca^{++} 添加実験の結果を，そのまま温血動物の心臓作用にあてはめることは不可能としても，Gottdenker らの実験成績とを考えあわせると，温血動物の心臓にたいする弗素イオンの特異な作用はあるていど推察しうるとおもわれる。ウサギ ECG にたいして 30mg/kg 静注では徐脈を，50mg/kg i.v. では S-T 下降，Q-T 延長にともなう抑制効果がみられた。いつぽう末梢血管にたいしては0.05%で著明な血管収縮を示すのにたいし，末梢作用を除外した血管中枢作用実験では 30 mg/kg i.v. ていどですでに注射直後から血管拡張作用をみとめ，50mg/kg i.v. ではさらに著明かつ持続的な血管拡張がみられた。血清 Ca，Ca^{++} 量は 30 mg/kg i.v. で下降し，注射後 1～4^h が最低値であつた。このさいのウサギの血圧は 30^m 前後が最低となり 60～90^m では全く回復しており，両者のピークは時間的に一致しなかつた。

これらの実験事実を考えあわせて，弗化ナトリウムの降圧作用機序は，たんに生体内の脱カルシウム作用の結果のみでは説明しえないことで，かなり強い血管中枢の抑制作用と心抑制作用とが直接の原因であると推察され，弗素イオンじしんのもの特性がより重要な役割を演じているものとおもわれる。

これにたいしクエン酸ナトリウムではカエル心にたいしては低濃度で著明な Positive inotropic action がみられ，高濃度では抑制作用がみられるが他の2者に比して最も弱く，摘出ヒキガエル心にたいする抑制効果も，Ca^{++} 添加によつてほとんど薬物じしんの影響は現れなかつた。ウサギ ECG ではクエン酸ナトリウム 120mg/kg i.v. でも，直後洞性徐脈をみるがまもなく回復し，30^m～1^h ころ軽度の Q-T 延長が現れるが，いずれも一過性で 2^h ごにはほとんど正常に回復した。血管運動中枢にたいしては，72mg/kg i.v. 以上で軽度一過性の抑制がみられ血管は拡張するが，まもなく回復する。末梢血管にたいしてはほとんど作用なく，わずかに拡張傾向がみられたにすぎなかつた。ウサギの血清 Ca，Ca^{++} は 72mg/kg i.v. で注射後 20～60^m が最低となり，2^h 以後には回復した例が多く，作用持続時間の短いことがうかがいしれた。

蓚酸ナトリウム高濃度にお[illegible]エル心抑制作用は3者中最も強いが，Ca^{++} 添加液[illegible]のヒキガエル摘[illegible]

心にたいしてはほとんど蓚酸ナトリウムじしんのもつ抑制効果がみられなかつたことは，クエン酸ナトリウムと同様に，弗化ナトリウムの場合とは全く異つた所見であつた。ウサギ ECG にたいしても，蓚酸ナトリウムのもつ心抑制効果は3者中最も強く，48mg/kg i.v.ですでに強い抑制効果をみ，その持続時間もきわめて長かつた。血管運動中枢にたいしては，注射後しばらくの潜伏期間を経てから徐々に抑制効果がみられ，程度ながら持続時間は長かつた，末梢血管にたいしてはクエン酸ナトリウムと同様ほとんど無作用であつた。ウサギ血清 Ca. Ca^{++} 量は 48mg/kg i.v.で注射後1～2^{h} で著明に下降し，下降度は他の2者に比し最も大であつた。血圧もこの量では60～9^{m} で回復する例は少なかつた。

以上の実験事実からクエン酸ナトリウム及び蓚酸ナトリウムの血圧下降も，心筋抑制と血管運動中枢の抑制が関与していることは推察されるが，Ca^{++} の変動が大きな因子として関与していることが推察され，この点今後の検討を要する。

これら3薬物はともに血圧下降とともに呼吸興奮が強く現れたが，これは薬物そのものが呼吸中枢を興奮するというよりは，血圧下降にともなう anoxia によつて反射性に起るものとおもわれる。しかしクエン酸ナトリウムの呼吸興奮は他の2者よりつねに著明にあらわれ，降圧作用がそのわりに小である点から，薬物じしんにも呼吸中枢の興奮作用があるものと想像される。

Schwyzer[8]は小量の弗素投与で著明な尿中 Ca 量，Cl 量の排出増加をみとめ，荻生もウサギに弗化ナトリウムを投与して尿中 Cl 量排出増加をみたと報告している。高森・川原[19]はウサギに弗化ナトリウム 10 mg/kg，30mg/kg を連日経口投与して，初期に尿素 Clearance の一時的増加と腎糸毬体の著明な充血を認め，時日の経過とともに蛋白尿，尿素 Clearance 値の減少，血清残余窒素および Creatinine の増量をみたと報告し，また川原[20]は 10mg/kg の弗化ナトリウムをウサギに静注して，FF が増加して糸毬体濾過機能の亢進を認めたが，30mg/kg 静注では GFR，FF が減少傾向を示し，病理組織学的に糸毬体腎炎の所見がみられたと報告している。

著者はウサギ橈尿管にカニューレを挿入し，薬物投与による尿量の増減を測定した。弗化ナトリウム10～20mg/kg 静注で，直後から6～7倍の著明な尿量増加をみ，30～40^{m} 持続した。しかし 50mg/kg 静注では直後一過性に尿量は増加するが，まもなく減少し全く停止することもあつた。小量の弗素投与による利尿効果は，川原の報告から考察すれば糸毬体濾過機能の亢進によるものとおもわれる。また弗素が強い酵素阻害作用をもつ点から，もしこの程度の量で炭酸脱水酵素を阻害するとすれば，この点からも利尿効果が起りうると推定されるが，今回の著者の実験ではその作用機序にたいして研究してないので，今後この方面の研究が必要とされる。

クエン酸ナトリウム (24～48mg/kg)，蓚酸ナトリウム (32mg/kg) でも注射直後一過性の尿量増加がみられた。Love[9]はこれら両薬物を 30mg/kg イヌに静注すると，腎血流量が増加すると報告しているが，然りとすればこれらの薬物によつて起る利尿効果は，腎血管拡張による血流量増加によるものとおもわれる。しかし3薬中著明な利尿効果のみられたものは弗化ナトリウムで，クエン酸ナトリウムは一過性であり，蓚酸ナトリウムは軽度ながら持続的であつた。

Heffter および Goodman & Gilman の各書によれば弗素は胃腸管の興奮性を亢進するといい，平田[21]は慢性弗素中毒ウサギの摘出腸管は，正常ウサギの摘出腸管より薬物にたいする感受性が亢進したと報告している。いつぽう Salant & Schwartze[22]はクエン酸ナトリウム・蓚酸ナトリウムの腸管にたいする作用を実験し，小量ではウサギ小腸運動が亢進し，大腸運動は抑制され，大量では大腸・小腸ともに運動は抑制されると報告し，Love はイヌについてはほ同様の効果をみとめ，Adrenaline 類似作用と報告している。

著者の実験成績では，ウサギ生体内小腸運動は，弗化ナトリウム 10mg/kg，クエン酸ナトリウム 24 mg/kg，蓚酸ナトリウム 16mg/kg i.v.についてみると弗化ナトリウムが最も小腸運動の亢進をきたし，振幅増大を示したが，クエン酸ナトリウムならびに蓚酸ナトリウムでは一過性緊張上昇のち低下して，運動振幅は軽度減少した。弗化ナトリウム 30mg/kg，クエン酸ナトリウム 72mg/kg および蓚酸ナトリウム 48 mg/kg 静注では，3者とも直後一過性緊張上昇を示したが，弗化ナトリウムはそのご振幅の軽度減少を示し，クエン酸ナトリウムは緊張は二相性に直後上昇のち低下を示したが，振幅にはほとんど変化なく，蓚酸ナトリウムは振幅律動数ともに減少した。

ウサギ摘出小腸運動は，弗化ナトリウム 10^{-6}，クエン酸ナトリウム 2.4×10^{-6}，蓚酸ナトリウム 1.6×10^{-6} 各溶液の流流では3者とも著明な振幅増大がみられた。弗化ナトリウム 10^{-8}，クエン酸ナトリウム 2.4×10^{-8}，蓚酸ナトリウム 1.6×10^{-8} では，3者とも律動は停止するが，弗化ナトリウムは緊張を著明に上昇せしめ Tyrode 交換後もさらに上昇した。クエン酸ナトリウムでは緊張低下のまま停止し，Tyrode 交換後さらに低下を示した。しかしそののちまもなく回復して律動を開始した。蓚酸ナトリウムは緊張上昇のまま停止したが，時間とともにやや低下傾向を示し，Tyrode 交換後さらに低下した。

以上のごとく，脱カルシウム作用を有するこれら3薬物について，各々同一 Equivalent concentration でその実験成績を比較検討した結果，多くの場合ほぼ類似の傾向を示しはしたが，作用時間や程度などには著しい差がみられ，とくに心臓作用にたいする Ca^{++} との関係などは全く異つた所見を示したものもあつた。

さらにこれら3薬物についての酵素阻害作用の面から，現在までの諸家の研究報告をみても，弗素の酵素阻害作用に関しては緒言で述べたごとく極めて多いが，クエン酸ならびに蓚酸ナトリウムの酵素系阻害作用を有するという文献は見当らない。著者の実験も今回は弗素の血液カタラーゼ阻害作用ならびに肝キサンチンオキシダーゼにたいする作用について行つたのみで，3者を比較検討することはできないが，個々の実験考察において述べたごとく，複雑な生体機構に作用する薬物の影響が，たんに血清電解質の一時的変動のみによつてもたらされるとは考え難く，このことは今回の実験成績の上からでもすでにいいうることで，今後酵素系にたいする阻害作用の面からこれらの薬物を比較すれば，さらに弗素イオンの特異性を摑みうることとおもうが，今回は主として一般薬理作用の面からのみの実験とその考察にとどめた。

ウ齒予防薬として現在最も有力な薬物である弗化物とくに弗化ナトリウムは，この方面において今後益々その用途は拡大されてゆくであろうが，このさいその薬理学的性質を明らかにしたことによつて基礎的知識の参考資料になれば幸甚である。

要　約

弗化ナトリウムの毒性ならびに一般薬理作用を，クエン酸ナトリウムおよび蓚酸ナトリウムと同一 Equivalent concentration において比較観察し，つぎの結論をえた。

1．弗化ナトリウムのマウスにたいする中毒症状として，興奮につづく麻痺がみられるが，クエン酸ナトリウムでは強い中枢興奮症状がみられ，蓚酸ナトリウムでは不穏状から鎮静状麻痺に移行した。弗化ナトリウムのマウス腹腔内注射による LD_{50} は 71.5～75.8 mg/kg，クエン酸ナトリウムの LD_{50} は 2.67～3.90 g/kg，蓚酸ナトリウムの LD_{50} は 345～385mg/kg であつた。

2．生体内カエル心にたいしては，弗化ナトリウムは0.2%，0.5ml/10g s.c. 以上で心運動の抑制がみられ，2%，0.5ml/10g s.c. では著明な振幅減少，ブロック，緊張低下を示し，同一 Equivalent 量の蓚酸ナトリウムもほぼ同様の経過をたどるが，クエン酸ナトリウムは緊張低下がみられるだけで，振幅，律動にはほとんど変化がなかつた。

3．摘出カエルおよびヒキガエル心にたいして，クエン酸ナトリウムは0.0048%前後で著明な振幅増大がみられたが，他の2者では一定の傾向がえられなかつた。弗化ナトリウム0.02%以上では著明な振幅減少，ブロックがみられ，蓚酸ナトリウムも同様の傾向を示したが，クエン酸ナトリウムの心抑制効果は3者中最も小であつた。

4．弗化ナトリウム0.02%溶液に脱カルシウム計算量の $CaCl_2$ を加えた溶液の灌流では，薬物単独灌流時よりややおくれて伝導障害があらわれるが，振幅減少はおこらなかつた。しかし同一 Equivalent 濃度のクエン酸ナトリウム，蓚酸トリウムはともに一過性の振幅減少がみられたにすぎなかつた。

5．ウサギ ECG にたいして，弗化ナトリウム 30 mg/kg i.v.では著変なく，同一 Equivalent 量のクエン酸ナトリウムも同様であつたが，蓚酸ナトリウムでは著明な S-T 下降，Q-T 延長などの抑制効果がみられ，2^{h} 以上を経過しても回復の傾向はみられなかつた。弗化ナトリウム 50mg/kg i.v.では不整脈，S-T 下降，T の平低化，Q-T 延長などがみられ，クエン酸ナトリウムは程度の Q-T 延長，頻脈につぐ徐脈がみられたが，2^{h} 後には回復した。

6．ウサギ血圧は各薬物によりいずれも下降を示すが，クエン酸ナトリウムは一過性，蓚酸ナトリウムは徐々に下降し持続時間は長かつた。弗化ナトリウムはほぼ両者の中間的作用を示した。

7．ウサギ呼吸は各薬物によりいずれも興奮を示すが，クエン酸ナトリウムは一過性著明，蓚酸ナトリウムは軽度であり，弗化ナトリウムは両者の中間的作用を示した。

8．ウサギ摘出耳介血管ならびにヒキガエル後肢血管にたいし，弗化ナトリウムは0.05%で著明な収縮を，2%で拡張をみるが，他の2者ではほとんど作用がなかつた。

9．血管中枢作用実験（神田法）では，各薬物によりいずれもウサギ耳介血管の拡張がみられるが，弗化ナトリウムは著明かつ持続的，クエン酸ナトリウムは軽度一過性，蓚酸ナトリウムは軽度[illegible]であつた。

10．ウサギ摘出腸管（Magnus 法）にたいし，各

物により，低濃度ではいずれも著明な振幅増大がみられるが，弗化ナトリウム0.02%で運動停止し緊張は著明に上昇した。クエン酸ナトリウムは緊張低下の傾向を示し，蓚酸ナトリウムは両者の中間的作用を示した。

11．ウサギ生体内腸管運動（Trendelenburg 法）は，弗化ナトリウム 10mg/kg i. v.では著明な振幅増大がみられたが，他の2者では注射直後一過性の緊張上昇のち低下がみられたのみで振幅には著変がなかつた。大量の静注ではいずれも振幅減少，緊張上昇がみられるが，弗化ナトリウムは著明で，他の2者は軽度であつた。

12．輸尿管カニューレ法によるウサギ利尿実験では，各薬物によりいずれも尿量増加がみられたが，弗化ナトリウムはとくに著明で，クエン酸ナトリウムは軽度一過性，蓚酸ナトリウムは軽度持続的であつた。

13．ラット呼吸ガス代謝（Knipping 型呼吸ガス代謝測定装置による）にたいして，弗化ナトリウム，蓚酸ナトリウムは O_2 消費量，CO_2 排出量の減少と体温下降がみられるが，クエン酸ナトリウムは逆に O_2 消費量，CO_2 排出量の増加がみられ，体温に著変はみられなかつた。

14．各薬物静注により，ウサギ血清総カルシウム，透析性カルシウムはいずれも減少を示すが，クエン酸ナトリウムは最もその程度が小であつた。弗化ナトリウムでは同時に血清総マグネシウムおよび無機燐が軽度増加の傾向を示したが，他の2者では一定の傾向がえられなかつた。

15．弗化ナトリウムは血液 Catalase による H_2O_2 分解能を低下せしめたが，Xanthine-Oxidase activity には明らかな抑制作用はみられなかつた。しかし Endogenous respiration (E.R.) にはすべての例で抑制傾向がみとめられた。

以上の実験成績からみて，同じ脱カルシウム作用を有するこれら3薬物の同一 Equivalent concentration の薬理作用にはそれぞれの差異がみられ，弗化ナトリウムの毒性をたんに脱カルシウム作用の面にのみ求めるべきでなく，弗素イオンじしんの作用にも意義のあることを考察した。

本論文の要旨は第22回日本薬理学会関東部会（昭和35年6月19日）において発表した。
稿を終るにあたり，ご指導と校閲を賜つた赤羽治郎教授に深謝します。

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SEQUENTIAL SCREENING: ITS ADAPTATION TO THE SEARCH FOR SUBSTANCES AFFECTING ACTIVITY OF KNOWN PHARMACOLOGICAL AGENTS[1]

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Received for publication March 21, 1961

The utilization of Wald's (1945) sequential analysis has greatly improved the design, efficiency and control of screening procedures, and descriptions of their useful applications have been made by several investigators (Sloboda *et al.*, 1957; Osterberg *et al.*, 1958; Dearborn, 1959; Cummings *et al.*, 1960). A review of these procedures, hereafter to be referred to as sequential screening, has been made by Litchfield (1960). These investigators were concerned with testing a compound(s) for the presence or absence of a particular pharmacological action following its administration to laboratory animals. For instance, Cummings *et al.* (1960) utilized sequential screening for the detection of compounds possessing diuretic activity. The procedure of Osterberg *et al.* (1958) was with reference to finding compounds which would abolish "paw-licking" of mice placed on a hot plate.

It is the purpose of this paper to show that sequential screening can be adapted to searches for substances which will increase or decrease a pharmacological effect of a known agent. In principle, this involves selection of a pharmacological effect and measurement of its variability, definition of significant effect, definition of error terms and probability of their occurrence and, finally, application of Wald's formulas for the derivation of the operating parameters of the test procedures (1945).

For illustrative purpose, we have chosen to describe the development and use of sequential screening for the detection of substances which, on co-administration with a tetracycline, might elicit blood tetracycline concentrations higher than would be expected if the antibiotic were administered alone. Tetracycline hydrochloride was selected as the representative of this class of antibiotics because apparently it is not metabolized in the rat and dog (Kelly and Buyske, 1960), it can be readily and cheaply labeled with tritium (Takesue *et al.*, 1960) and a radiometric assay suitable for carrying out large numbers of determinations is available (Takesue *et al.*, 1960).

The general method of approach, however, can readily be adapted to a search for substances which enhance or inhibit other types of pharmacological activities.

SOURCE OF DATA. Orally administered tritiated tetracycline plus citric acid, a substance known to increase the serum concentrations of tetracycline, and tritiated tetracycline alone constituted the standard and control substances, respectively. Male Wistar rats, weighing between 180 and 230 g, were used. The animals, fasted overnight, received *per os* 50 mg/kg of tritiated tetracycline either alone or with 50, 100, 150 or 200 mg/kg of citric acid. Tritiated tetracycline alone or in combination with citric acid was administered as an aqueous solution at a dose of 1.0 ml per 100 g of rat body weight. Blood samples from each rat were collected at 2, 4 and 24 hours following administration and serums assayed as previously described (Takesue *et al.*, 1960). The experiment was repeated three times, one week apart. Each time, a new group of rats was used.

RESULTS. *Analysis of data.* To facilitate statistical analysis, the three values for each rat were reduced to a single one by calculating the areas under the curve (AUC) as shown in figure 1. If M, N and P are the respective 2-, 4- and 24-hour serum concentrations of tetracycline for one animal, a single result can be obtained by geometrically calculating the areas

[1] A portion of this material was presented at the Fall Meeting of the American Society for Pharmacology and Experimental Therapeutics, Seattle, Washington, August 1960.

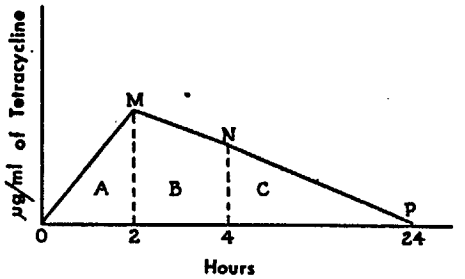

FIG. 1. Calculation of area under curve (AUC), where M, N and P represent the 2-, and 4- and 24-hour concentrations of tetracycline, respectively, from geometric formula:

$$AUC = A + B + C = \frac{2M}{2} + \frac{2(M + N)}{2} + \frac{20(N + P)}{2} = 2M + 11N + 10P$$

A + B + C and subsequent reduction to the formula 2M + 11N + 10P. Substitution and development yield the area under curve value which represents μg hours of tritiated tetracycline per ml of serum.

The data, so transformed, are given in table 1. A preliminary analysis revealed that the variance associated with each of the 15 groups did not differ significantly one from the other, *i.e.*, the range of the responses for each group was not affected either by treatment (different doses of citric acid) or by the time factor (three experiments, each conducted 1 week apart).

The determination of the effects of citric acid upon absorption of tritiated tetracycline at any given week, the reproducibility of the results from week to week and other related findings were assessed *via* an analysis of variance. The results are summarized as follows. 1. At any given week: a. The administration of 50 mg/kg of citric acid with 50 mg/kg of tritiated tetracycline did not result in an area under the curve significantly greater than that obtained following administration of tritiated tetracycline alone. b. Administration of 100, 150 or 200 mg/kg of citric acid with 50 mg/kg of tritiated tetracycline resulted in areas under the curve significantly greater than that of the control group. 2. *From week to week:* a. The areas under the curve of treatment groups receiving 50 mg/kg of tritiated tetracycline alone differed significantly; this was also true for the areas under the curve of animals receiving 50 mg/kg of citric acid and tetracycline. b. There were no significant differences in the areas under the curve of groups receiving 100 mg/kg of citric acid with 50 mg/kg of tetracycline. The respective responses of the groups receiving 150 or 200 mg/kg of citric acid were also reproducible from week to week.

TABLE 1

Source of data for development of sequential procedure

Areas under curve (average) following a single oral administration of 50 mg/kg of tritiated tetracycline hydrochloride and added amounts of citric acid to rats.*

Amount of Citric Acid (mg/kg)	μg hour/ml of Serum (Tritiated Tetracycline Hydrochloride)		
	Expt. I	Expt. II	Expt. III
none	82.2	84.8	53.1
50	91.7	86.1	61.6
100	120.3	120.8	78.3
150	173.2	139.4	138.2
200	169.2	187.1	154.1

* Number of animals per treatment: controls 8; treatments 4.

Further investigation revealed that it would be possible to eliminate the 2-hour value and calculate the area under the curve from the 4- and 24-hour values only and not invalidate any of the findings obtained. Since, with respect to sample collection, this would further simplify the procedure, it was decided to carry out the development of the sequential analysis on this basis.

An additional finding was the increase in areas under the curve in relation to the amounts of citric acid. Calculation of the regression line for each experiment showed that the three did not differ significantly with respect to slope or position. Graphic representation of the regression lines is shown in figure 2. It should be noted that rather than absolute areas under the curve, the ordinate expresses the differences between the areas under the curve of groups receiving tetracycline plus citric acid and of those receiving tetracycline alone.

Development of the sequential screening procedure. The data on hand represented the fundaments from which the parameters of the pro-

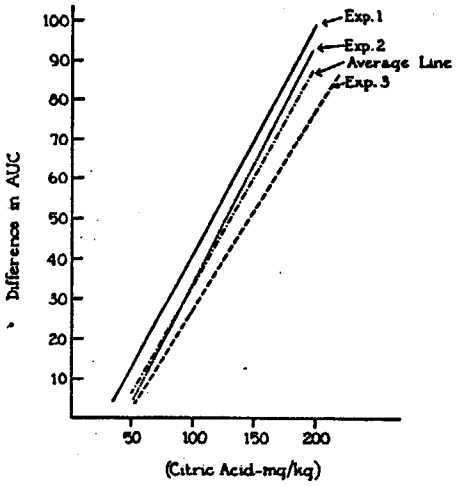

FIG. 2. Calculated regression lines of three experiments.
Effects of increasing amounts of citric acid on differences between AUC of groups receiving tetracycline plus citric acid and AUC of groups receiving tetracycline alone.

dure were to be drawn. However, since the data had been obtained from three experiments only, it had to be assumed that this small sample was truly representative of an infinite number of experiments similarly conducted. (The proof of such an hypothesis will be shown later by subsequent data.) Together with this assumption, certain definitions and choices had to be made in order to calculate the parameters of the sequential procedure. These were: 1. A substance would be considered "good" when, following its administration with tetracycline, the response (area under the curve) was similar to that of tetracycline plus citric acid. A "poor" substance was one which would elicit a response similar to that of tetracycline alone. 2. The doses of tetracycline would be 50 mg/kg. The doses of citric acid or unknown substances would be 140 mg/kg. (Lower doses of citric acid were not satisfactory since, in view of the small effect, an impractical number of animals would be needed to show a response significantly different ($P = 0.01$) from that of tetracycline alone.) At these doses, the area under the curve for tetracycline alone was 68 ± 4.3 while that for the tetracycline-citric acid combination was 130 ± 4.7 μg hr/ml (average value ± standard error of the mean).

3. Contrary to the usual nonstatistical procedures, the chances of error occurrence in judging a compound can be, theoretically, preselected; these were set as follows: the probability of calling a "truly poor" substance "good" was set at 0.01 (α error); the probability of rejecting a "truly good" substance was also set at 0.01 (β error); the probability, therefore, of accepting a "truly good" substance was 0.99 ($1-\beta$).

The above data and specifications, with the estimated standard deviation, were substituted into Wald's (1945) formulas and the parameters for a three-stage sequential screening plan were obtained (table 2).

It will be noted that when an unknown substance(s) is first tested, the plan will either reject, accept or call for a retest according to the critical values of Stage I. If, for example, the area under the curve for tritiated tetracycline and unknown was 100 and that of tritiated tetracycline plus citric acid was 130, since the difference ($100 - 130 = -30$) was between the critical values of acceptance (-3.9) and rejection (-56.1) of Stage I, the unknown would be retested. Following retesting, the arithmetic average of the results from both experiments and the Stage II critical values are used. If, in the first stage, the difference had been greater than -56.1 or less than -3.9, the substance would be rejected or accepted accordingly. In the rare occurrence that a substance had to be tested three times, it would be accepted or rejected on the basis of the value of Stage III.

The theoretical probability of acceptance of compounds according to this plan was calculated and is given in figure 3. It will be noted that the curve, known as the operating characteristic

TABLE 2

Parameters for acceptance or rejection of substances affecting absorption of tetracycline by a three-stage sequential screening procedure

Stage	Differences in AUC*	
	Reject	Accept
I	≤ -56.1	≥ -3.9
II	≤ -43.1	≥ -16.9
III	≤ -30.0	> -30.0

* Mean AUC of 3 rats receiving unknown and tetracycline minus mean AUC of 3 rats receiving citric acid and tetracycline.

curve of the plan, expresses all degrees of differences and related probability of acceptance. It is apparent that the smaller the differences, the greater the probability of acceptance.

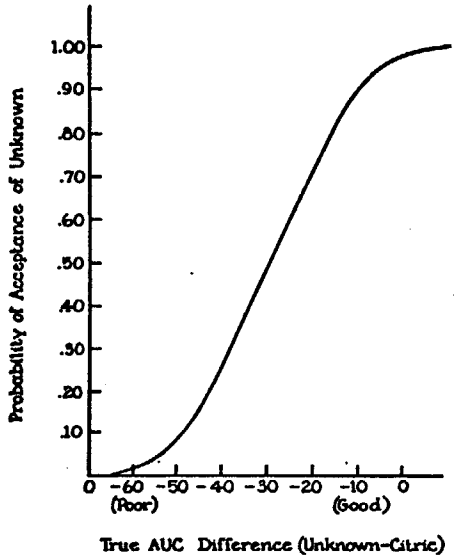

FIG. 3. Operating characteristic curve.
Theoretical probability of acceptance based on true differences between AUC of unknown plus tetracycline and AUC of citric acid plus tetracycline.

The next figures constitute an important part of the plan. To ascertain the correctness of the characteristics of the sequential screen which, as previously mentioned, has been derived from three experiments, control charts were maintained. The functions of these charts were to determine not only whether in subsequent experiments the tetracycline alone and tetracycline plus citric acid groups were functioning as predicted, but also whether the additional data accumulated for both might permit further simplifications or call for a revision of the plan. The control charts and the responses of the two groups in eleven experiments are given in figures 4A and B and 5.

Figure 4A shows the average area under the curve of rats receiving tritiated tetracycline alone. The straight solid and dashed lines represent the average and the upper and lower limits (three standard deviations) of the first three experiments. The dots represent the responses of the groups given tritiated tetracycline alone in subsequent experiments. As is apparent, the control groups were running as predicted, since the responses are well within the limits, as shown by running average (uneven line) (Askovitz, 1955).

Figure 4B represents the average areas under the curve for the groups of rats receiving tritiated tetracycline and citric acid. The dots are the results of the subsequent individua

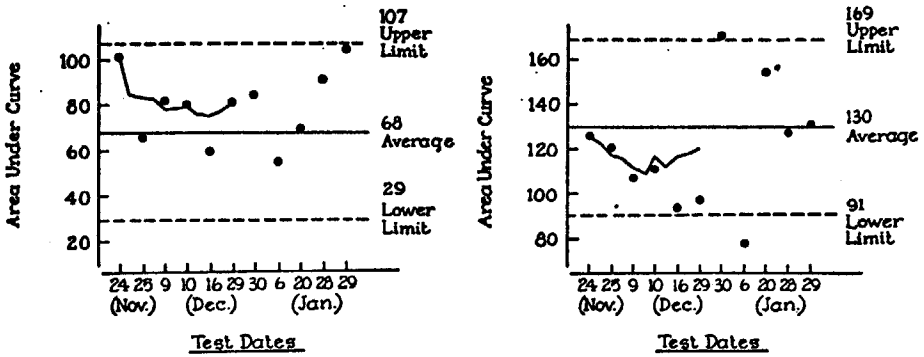

FIG. 4A (*left*). Control chart: Average AUC of groups receiving 50 mg/kg of tetracycline alone.
Straight solid and dash lines represent the average AUC and 3 standard deviations of first three experiments. Dots represent average AUC of same treatment groups in subsequent experiments, the uneven line is a running average.
FIG. 4B (*right*). Control chart: Average AUC of groups receiving 50 mg/kg of tetracycline plus 140 mg/kg of citric acid.
Straight solid and dash lines represent the average AUC and 3 standard deviations of first three experiments. Dots represent average AUC of same treatment groups in subsequent experiments. The uneven line is a running average.

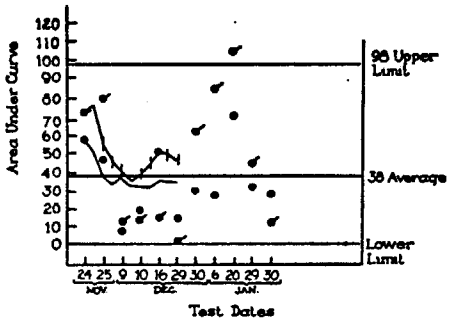

FIG. 5. Control chart: Ranges of AUC of the two treatment groups as indicated by straight solid and dash lines in the first three experiments. The dots represent the range of AUC of groups receiving 50 mg/kg of tetracycline alone (●) and of groups receiving 50 mg/kg of tetracycline plus 140 mg/kg of citric acid (♦) in the subsequent experiments; the respective running averages (uneven lines) are indicated by the solid angular line and the solid line with vertical crossbars, respectively.

experiments while the straight solid and dashed lines, as mentioned previously, are the results (average and upper and lower limits) obtained from the three initial experiments. As indicated by the running average (uneven line), the responses, although limited in number, showed no definite trend. It is apparent, however, that in two experiments the standard responses were outside the permissible limits. This occurrence in 2 of 11 experiments, which far exceeded the expected 3 in 1000 frequency,[2] indicated that no decision could be made on the concomitantly tested compounds, since the conditions of the sequential screen were not under "control." A retest, therefore, became necessary.

Figure 5 graphically depicts the experimental variations of the two treatment groups as indicated by the respective ranges of areas under the curve. It will be noted that the values were within the expected limits.

As mentioned, it became feasible, at this point, to re-evaluate all of the compiled data in the hope of further simplifying the test procedure.

It was found that the responses of the groups given tritiated tetracycline alone in the first three experiments did not differ significantly from those obtained in the eleven subsequent experiments. The same conclusions were reached when the tritiated tetracycline plus citric acid groups

[2] Derived from 3 standard deviations (997/1000).

TABLE 3

Parameters for acceptance or rejection of substances affecting absorption of tetracycline by a three-stage sequential screening procedure

Stage No.	Differences in μg/ml of Serum*	
	Reject	Accept
I	≤−3.62	≥0.14
II	≤−2.82	≥−0.80
III	≤−1.74	>−1.74

* Serum concentrations of unknown and tetracycline minus serum concentrations of citric acid and tetracycline at 4 hours after dosing.

were analyzed. A similar comparison based on the 4-hour serum tetracycline concentrations led to the same conclusions. In addition, the acceptance or rejection of compounds tested was unaffected. It was, therefore, possible to omit the 24-hour serum samples thus further simplifying the procedure.

These findings, together with the smaller variances, allowed the calculation of a simpler sequential analysis, which is presented in table 3. It will be noted that, rather than AUC values, the actual concentration of tetracycline, in μg/ml of serum, is now used. The definition of a good or poor compound remains unchanged.

On the basis of these new parameters, only two animals per stage are needed to reach a decision for any unknown substance. Eighty-two per cent of the test substances will be disposed of after the first stage, a second or third stage being necessary for the remainder. The small variance and the increase (from 3 to 6) in number of rats receiving the standard compound made this possible. The calculated risks (α and β) and doses of standard and control groups were not changed. The operating characteristic curve and the control charts, basically similar to those previously presented, are omitted here for brevity.

Testing for known compounds. In order to determine the effectiveness of the test, compounds which had been reported to increase the blood concentration of tetracyclines were tested by the sequential procedure. The results are given in table 4. As is apparent all of the compounds were accepted.

DISCUSSION. Although quantitative testing is possible, the primary purpose and merit of sequential screening is that it permits rapid differentiation of tested substances into either a "of interest" class, for follow-up studies, or a "no interest" class. This is an important point for the investigator using screening techniques since he is confronted with the problem of how to test quickly and reliably a significant portion of many compounds available. This and other aspects, such as the rationale of sequential screening in general, its advantages over other types of screening procedures with respect to better utilization of animals, compounds and time and some of the objections to the method, have been presented in part by Dearborn (1959) and Cummings *et al.* (1960) and extensively by Litchfield (1960).

TABLE 4

Decisions on commercial or reported substances by sequential screen following single oral administration to fasted rats at 1:2.8 w/w ratio (tetracycline: 50 mg/kg; substance: 140 mg/kg)

Substance	Decision		
	Stage I	Stage II	Stage III
Terephthalic acid	Cont.	Cont.	Accept
Glucosamine	Cont.	Cont.	Accept
Na metaphosphate	Cont.	Cont.	Accept
Na phosphate	Accept		
Na pyrophosphate	Cont.	Cont.	Accept
Na glycerophate	Cont.	Cont.	Accept
Monosodium citrate	Cont.	Cont.	Accept
Trisodium citrate	Cont.	Accept	
Phytic acid	Accept		
Na—phytate	Cont.	Accept	
Na—EDTA	Accept		
Ascorbic acid	Accept		
Lactic acid	Cont.	Cont.	Accept
Malonic acid	Accept		
Malic acid	Accept		
Mucic acid	Cont.	Cont.	Accept
Tartaric acid	Accept		
Tricarballylic acid	Cont.	Cont.	Accept

We will limit the discussion to the following aspects: a) the use of standards for the derivation of the parameters of activity, and b) the use of labeled materials in sequential screening.

With reference to the first aspect, the investigator must decide what will constitute a significant response on the part of the unknown substance for its acceptance. This is true regardless of whether the procedure to be developed is concerned with finding new pharmacological agents, as in the case of the previous investigators, or with finding new substances affecting activity of known pharmacological agents, as in our case. The criteria of activity can be derived either by using the responses of untreated animals or the responses elicited by a standard. Cummings *et al.* (1960), for example, used the 5-hour urinary output of untreated animals; he could have used equally as well the urinary output elicited by a known diuretic. We have used the responses to a standard, namely, citric acid; we could have used the data obtained from the rats receiving tetracycline alone.

The use either of values from untreated animals or of the responses to the pharmacological agent alone—as in cases similar to ours—may lead to the following: the investigator might find that, on the basis of data analyzed, a 20% change is statistically significant and therefore decide to use this finding for the derivation of the criteria of activity. The number of substances being accepted, however, may be so large as to hinder seriously follow-up studies. On the other hand, he might arbitrarily set a 200% change from the norm as a critical value for acceptance. Such a selection might result in rejection of truly valuable substances. Such occurrences, instead, might be reduced if an appropriate standard is used, since the definition of what will constitute a significant effect on the part of the unknown substances can be based, at least in part, on what information is available on the standard. For instance, the effects of citric acid, glucosamine, sodium metaphosphate and terephthalic acid on serum concentrations of co-administered tetracyclines have been studied in man and animals (Carlozzi, 1958; Dearborn *et al.*, 1957; Eisner *et al.*, 1953; English, 1958; Gray *et al.*, 1954; Peterson and Hendrix, 1959; Shelton and Olson, 1958; Sweeney *et al.*, 1957; Welch *et al.*, 1957a–c; Welch and Wright, 1957; Welch *et al.*, 1958). With the exception of the terephthalic acid–chlortetracycline combinations in certain veterinary diseases (Peterson and Hendrix, 1959; Shelton and Olson, 1958), the effects of the substances have neither justified a reduction in dosage nor elicited superior clinical effects with respect to the antibiotic used.

From the foregoing, it is obvious that the procedure should be constructed to detect substances whose effects are at least equal to those elicited

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by citric acid. (The use of this standard, in addition, will eliminate from follow-up studies those substances which, although not as good as citric acid, might still be significantly better than the control, tetracycline alone.) Although the majority of substances accepted will not differ markedly from citric acid, some, whose effects will be greater than the standard, will be of particular interest. Our findings, with respect to new agents of this type found by use of the sequential procedure, will be the subject of a forthcoming publication.

The second aspect we wish to discuss is the utilization of radioactive pharmacological agents in screening procedures. At times the search for substances which enhance or inhibit the activity of a pharmacological agent is not feasible since measurements of the latter are either difficult, impractical or nonexistent. If, for instance, we were to search for a substance affecting a hypotensive agent, the criteria of activity would be based on the fall in blood pressure and, consequently, the number of substances which could be screened, under such circumstances, would be limited. However, if the hypotensive agent could be tritiated or made radioactive otherwise, blood concentrations of the agent, as determined radiometrically, could be used for the construction of the procedure, rather than fall in blood pressure. Understandably, in such a case, there should be evidence that fall in blood pressure is directly related to blood concentrations of the agent.

A less complicated example to which sequential screening can be applied is that of dietary iron. It is known that this essential element is poorly absorbed when administered orally and blood determinations of the same are not only relatively difficult but are complicated by the existing iron in blood. The efficacy of compounds could be established by coadministration of radioactive iron and measuring the amounts of the latter appearing in blood.

SUMMARY

A description of the development and use of a sequential screening procedure for the detection of substances affecting activity of known pharmacological agents has been presented. For illustrative purpose, the procedure has been adapted to the screening of substances which will increase the blood concentrations of tetracycline following their concomitant oral administration with the antibiotic. The resultant criteria for acceptance or rejection of the tested substances are based upon the 4-hour serum concentration of a standard (tritiated tetracycline plus citric acid) and those of tritiated tetracycline plus the test substances. The antibiotic (50 mg/kg) and citric acid, or test substances, at 140 mg/kg, are administered orally to overnight fasted Wistar rats. At these doses, the average (± standard error) tetracycline serum concentrations for the control (tetracycline alone) and standard groups are 5.09 (±0.41), and 8.56 (±0.42) μg/ml. Eighty-two per cent of the test substances are disposed of after the first stage (two animals per stage). For the remaining, a second or third stage is necessary. Control charts are maintained to, a) ascertain that, in each experiment, the responses of the control and standard groups are within tolerable limits, and, b) detect any general trend, over several consecutive experiments, of the control and standard groups.

Acknowledgment. The authors wish to express their appreciation to Dr. Earl H. Dearborn for his interest in this study and to Mr. J. Haynes of the Statistical Analysis and Design Group for his guidance and counseling in the development and execution of this sequential plan.

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Annales pharmaceutiques françaises 1968, 26, No. 9-10, 593-598

Original Papers

Influence of citrates on renal lithiasis under ethylene glycol

By Ch. Vaille, Ch. Debray, M. Souchard and Cl. Roze (*)

Introduction

The oral administration of ethylene glycol in the male rate produces renal lesions characterized by crystallizations of calcium oxalate monohydrate (20). The females are much less sensitive to this toxin, and the administration of estrogens to male rats protects them against the appearance of renal concretions (15, 17, 18, 19).

We have investigated the effects of the citrate ion on experimental oxalosis of the rat, because on the one hand this acid plays an important part in the calcium metabolism (12) and, on the other, the estrogens cause an increase in the production and oxidation of the blood citrate (16), so that an intermediate effect of this ion in the action of the estrogens on renal oxalosis can be envisaged.

Experimentation

Four lots of ten male Wistar rats (G.N.R.S., Gif) were treated as follows:

1. Controls:

   Food in powder form: "Complete cooked food for rats UAR" (1*)

   Drink: water, ad libitum

2. Ethylene glycol:

   Food in powder form: normal.

   Drink: ethylene glycol at 1% in water.

3. Citrate:

   Food in powder form, prepared as follows:

Complete food	800 g
Citric acid	50 g
Monopotassium citrate (2 H_2O)	75 g
Monosodium citrate (1 H_2O)	75 g

Drink: water

4. Citrate + ethylene glycol:

Citrated diet

Drink: ethylene glycol at 1%.

After one week of observation, during which all animals were given the normal diet, lots 3 and 4 receive for one week the citrated diet alone to avoid a sudden change of diet. Then only ethylene glycol is administered to lots 2 and 4. At the end of three weeks after ethylene glycol, the animals are killed by bleeding the abdominal aorta under ether anesthesia. We determine the urea and the proteins in the serum; we take out and weight the following organs: kidneys, liver, spleen, adrenals, heart, a fragment of which is fixed in 10% formol for histologic examination. During the entire experiment the fluid intake is noted every day, and the weight of the animals measured twice a week. The consumption of citrates is estimated at about 2.5 g per rat and per day.

Results

1. Growth (Table I). - This was an experiment made on animals of rather heavy weight at the start, growing slowly. The results are marked, therefore, by a loss of weight, rather than a slower growth as is the case with younger animals. At the beginning of observation and at the beginning of treatment with citrate, all lots are of homogeneous weight. The citrated diet immediately gives a definite loss of weight, no doubt partially due to the change in calory intake,as this loss of weight then tends to level off. Also the ethylene glycol produces a very significant loss of weight. In the combination ethylene glycol / citrate, the effects of the two factors are not additive, that is, the loss of weight is not greater than with each of the two factors employed alone. The calculation indicates a very significant negative interaction.

2. Fluid intake (Table I).- The quantity of beverage absorbed on the average per rat and per day is substantially the same in the four groups. This, no doubt, has to do with the age of the animals, because in young rats ethylene glycol generally increases the amount of fluid taken.

3. Weight of the organs (Table II).- The spleen, heart, adrenals are not significantly changed, at least when taking into account the body weight of the animals. On the other hand, the kidneys show particularly interesting changes: Citrate alone does not modify their weight, whereas ethylene glycol alone clearly increases it, as is common. With the combination citrate + ethylene glycol, the weight of the kidneys is substantially normal, indicating a significant negative interaction between citrate and ethylene glycol. Also in the liver an effect is noted: Ethylene glycol and citrate both reduce the weight of this organ, more so in the case of the citrate. The combination shows additive effects, not interaction.

Table I. Interaction between ethylene glycol and citrate at forced doses in the rat. Fluid intake and growth.

	Control	E.G.	Citrate	Citrate + E.G.	Difference between groups	E.G. alone	Citrate alone	Interaction
Fluid (ml/rat/day)	23.8	25.1	28.9	29.1	N.S.			
Weight at start of observation (g)	435	422	452	454	N.S.			
Weight at start of citrate (g)	446	434	460	456	N.S.			
Weight at start of E.G. (g)	454	443	409	408	N.S. (0.10)			
Weight on sacrifice (g)	464	380	370	397	0.01	↓ 0.001	↓0.001	(-).001

Table II. Interaction between ethylene glycol and citrate at forced doses in the rat. Weights of the organs, urea, and serum proteins.

	Control	E.G.	Citrate	Citrate + E.G.	Diff. between groups	E.G. alone	Citrate alone	Interaction
Wt. of spleen (1) (mg)	593	561	579	597	N.S.	-	-	-
Wt. of heart (mg)	919	977	974	965	N.S.	-	-	-
Wt. kidneys (cg)	188	284	208	217	0.01	↗0.01	N.S.	(-) 0.05

Table II continued

	Control	E.G.	Citrate	Citrate + E.G.	Diff. between groups	E.G. alone	Citrate alone	Inter-action
Wt. liver (cg)	1116	954	872	792	0.001	0.05	0.001	N.S.
Wt. adrenals (mg)	48.5	48.5	45.0	41.8	N.S.	-	-	-
Urea (2) (cg/l)	44	288	47	47	0.001	0.001	N.S.	(-)0.001
Serum proteins (g/l)	64.8	66.1	65.9	71.3	N.S.	-	-	-

(1) Except in the case of the adrenals, the significance tests are calculated on the weights adjusted on ordinator according to the regression line organ weight/body weight calculated for the controls. The mean weights indicated are the adjusted values.

(2) Significance tests effected after logarithmic transformation.

4. Urea and serum proteins (Table II). The level of blood urea, which is clearly increased by E.G., remains normal only in the case of the combination ethylene glycol + citrate, confirming a strong negative interaction in the kidney and agreeing perfectly with the values of organ weights reported above. The serum proteins are not modified.

5. Histology. The renal lithiasis, the intensity of which is represented by + signs according to a previously described scale (2), is very great in the ten rats treated with ethylene glycol alone: One rat has a lithiasis of ++++, 2 rats of +++, 2 rats of ++, 5 rats of +. It is accompanied by a tubular nephritis in all the intoxicated animals. On the other hand, in the rats treated with citrate combined with ethylene glycol, there is lithiasis only in 4 rats: One rat at +, three others ±; in two of these lithiasic animals the calcium oxalate crystals are accompanied by a slight tubular dilatation and a slight epithelial atrophy, while the other two have no epithelial modifications of the tubes, just as little as do the non-lithiasic rats. The other series of rats have no histological modification of the kidneys. In all series the other organs are substantially normal.

Discussion

The citrates given at strong doses by mouth permit counteracting the nephritis

and the precipitation of calcium oxalate produced by ethylene glycol in the male rat. It is worth mentioning the cause and consequences of this action.

1. Calcium metabolism.- One of the hypotheses envisaged to understand how the calcium salts of the bones are dissolved and pass into the blood is that the citrate forming in the Krebs cycle is the chelating agent in question. The latter would intervene in the equilibrium between the mineral elements of the extracellular liquid and the mineral elements of the bone, with intervention of the parathormone, which would act on the bones through the citric ion to release the calcium (8, 13). One may wonder wheter the increase of the citrate ions in the organism prevents the precipitation of the oxalate in the form of calcium salt in the kidneys as a result of the chelation of the calcium. The calcemia may, in fact, increase in the course of ethylene glycol nephritis (3). Several factors are opposed to this hypothesis. We have shown that the calcemia increases the renal lithiasis from ethylene glycol only when there is a strong intoxication by the dialcohol alone; besides, we have been able to dissociate the lithiasis itself from the hypercalcemia in some experiment series (21).

Moreover, the Harrisons (7) have shown that oral administration of citric acid did not increase the degree of the citremia. Lichtwitz and his associates (15) have summarized the state of the question in these terms: "... the exogenic citric acid passes through the stomach without notable modifications, but it is destroyed later - either during its passage through the intestines, where it is metabolized in the Krebs cycle of the cells of the wall, or in the liver, as the supra-hepatic vein contains less citric acid than the vena portae... It seems, however, that this exogenic citric acid plays an important part in the intestinal absorption of the calcium", in the sense of an augmentation.

Lastly Elliot et al. (6) believe that the role of the citric acid in the normal urinary excretion of the calcium is not very important and practically insignificant in the lithiasis.

As this first hypothesis must be discarded, that of an intervention in the metabolism of the dialcohol remains to be considered.

2. Metalbolism of the ethylene glycol.- Lithiasic intoxication by ethylene

glycol is due essentially to its metabolism leading to the formation of oxalic acid. We have previously put forth the hypothesis that the reduction of oxalosis by the estrogens (19), by phenacitin (21), or by ethanol (4) could be explained by an enzymatic competition where NAD and probably NADP would intervene as coenzyme. Now, while the production of the endogenic citrate depends mostly on NAD, its subsequent oxidation requires NADP (9). It is conceivable, therefore, that the addition per os of strong doses of citrates can cause, either in the intestin or in the liver and probably in both, a strong increase of the coenzyme requirements in the Krebs cycle. Thereby the less metabolized ethylene glycol would be eliminated as such and without producing oxalic acid, hence lithiase.

3. Treatment of acute ethylene-glycol intoxications.- We have suggested, in the case of such an intoxication, to try blocking the enzymes of degradation of the toxin by intercepting them with ethyl alcohol, owing to ethanol perfusions in glucose solution for example (4). Peterson (14) and his associates have increased the LD_{50} of ethylene glycol in the rat by intraperitoneal injection of ethanol.

Wacker et al. (22), following up the results obtained in the treatment of intoxications by methyl alcohol, publish the result of their treatment of ethylene glycol intoxications with ethanol. In France, a demonstration of the fortunate results of this method was made at the Saint Vincent de Paul Hospital in Paris (10, 11). As the doses of ethyl alcohol necessary for treating the ethylene glycol intoxication may be large and an ethyl coma must obviously be avoided, we suggest trying to supplement this detoxication by intravenous ethanol by the administration of strong oral doses of citrates.

4. Treatment of lithiassis by citrates.- Cloarec and Rouffy (1) have treated patients with a strong dose of citric acid and citrates in order to obtain a medicamentous chemolysis of uric lithiasis. They were then able to observe a significant lowering of the concentrations of cholesterol, triglycerides, urea and uric acid in the blood. When Eisenberg, Connord and Howard recommended, in 1955, the use of such a therapy in the treatment of lithiasis, they took as a basis for doing so the hypothesis of a dissolution of the calculi by modification of the urinary pH. If one considers the previous studies on the hyperuricemizing action

of ethanol observed by Delbarre (5), it is permissible to suppose that the activity in the lithiasis of the citrates at strong doses is more likely attributable to a stimulation of the enzymes of the Krebs cycle. In fact, the citrate mixture here used changes but little the urinary pH in the rat, less than sodium bicarbonate at equal doses, and less than Eisenberg's solution, which contains trisodium and tripotassium citrates.

The administration of strong doses of citrate ion in male rats treated with ethylene glycol counteracts the formation of calcium oxalate lithiasis normally obtained under the effect of the dialcohol alone. Also tubular nephritis is prevented, which follos the crystallized deposits in the kidney. This experimentation explains the results obtained with the use of citrates in the therapy of lithiasis. This spectacular action suggests that the use of citrates in strong oral doses may be recommended in the treatment of acute ethylene glycol intoxication.

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(Footnotes)

(*) Paper submitted to the Academy of Pharmacy, session of May 8, 1968.

(**) Project sponsored by the I.N.S.E.R.M.

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1968, 26, n° 9-10, pp. 593-599

MÉMOIRES ORIGINAUX

Influence des citrates sur la lithiase rénale à l'éthylène-glycol,

par

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Introduction

L'administration orale d'éthylène-glycol chez le Rat mâle produit des lésions rénales caractérisées par des cristallisations d'oxalate de calcium monohydraté [20]. Les femelles sont beaucoup moins sensibles à ce toxique, et l'administration d'œstrogènes aux rats mâles les protège contre l'apparition des concrétions rénales [15, 17, 18, 19].

Nous avons recherché les effets de l'ion citrate sur l'oxalose expérimentale du Rat, car d'une part cet acide joue un rôle important dans le métabolisme du calcium [12] et, d'autre part, les œstrogènes provoquent à la fois une augmentation de la production et de l'oxydation du citrate sanguin [16], permettant ainsi d'envisager un effet intermédiaire de cet ion dans l'action des œstrogènes sur l'oxalose rénale.

Expérimentation

Quatre lots de dix rats mâles de souche Wistar (G. N. R. S., Gif) ont été traités de la manière suivante :

1° Témoins :

Nourriture en poudre : « aliment cuit complet pour rats UAR » (1).

Boisson : eau, *ad libitum*.

(*) Mémoire présenté à l'Académie de Pharmacie, séance du 8 mai 1968.
(**) Travail réalisé avec l'aide de l'I. N. S. E. R. M.
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2° Éthylène-glycol :

Nourriture en poudre : normale.

Boisson : éthylène-glycol à 1 % dans l'eau.

3° Citrate :

Nourriture en poudre, préparée comme suit :

Aliment complet	800 g
Acide citrique	50 g
Citrate monopotassique (à 2 H_2O)	75 g
Citrate monosodique (à 1 H_2O)	75 g

Boisson : eau.

4° Citrate + éthylène-glycol :

Régime citraté.

Boisson : éthylène-glycol à 1%.

Après une semaine d'observation, pendant laquelle tous les animaux sont soumis au régime normal, les lots 3 et 4 reçoivent pendant une semaine le régime citraté seul pour éviter un changement brutal de régime. A ce moment seulement l'éthylène-glycol est administré aux lots 2 et 4. Au bout de trois semaines après éthylène-glycol, les animaux sont tués par saignée à l'aorte abdominale sous anesthésie à l'éther. Nous dosons l'urée et les protides dans le sérum ; nous prélevons et pesons les organes suivants : reins, foie, rate, surrénales, cœur dont un fragment est fixé au formol 10 % pour l'examen histologique. Pendant toute la durée de l'expérience, la consommation de boisson est notée tous les jours, et le poids des animaux mesuré deux fois par semaine. La consommation de citrates est estimée à 2,5 g environ par rat et par jour.

Résultats

1° *Croissance* (tableau I). — Il s'agit d'une expérience conduite sur des animaux de poids assez fort au départ, dont la croissance est lente. Les résultats se marqueront donc par un amaigrissement, au lieu d'un ralentissement de croissance, comme c'est le cas

Tableau I. — *Interaction entre l'éthylène-glycol et le citrate à doses forcées chez le Rat. Boisson et croissance.*

	Témoins	E.G.	Citrate	Citrate + E.G.	Différence entre groupes	E.G. seul	Citrate seul	Interaction
Boisson (ml/rat/jour)	23,8	25,1	28,9	29,1	N.S.			
Poids début observation (g)	435	422	452	454	N.S.			
Poids début citrate (g)	446	434	460	456	N.S.			
Poids début E. G. (g)	454	443	409	408	N.S. (0,10)			
Poids sacrifice (g)	464	380	370	397	0,01	↘ 0,001	↘ 0,001	(—) 0,001

pour les animaux plus jeunes. En début d'observation et en début de traitement au citrate, tous les lots sont de poids homogène. Le régime citraté donne immédiatement un amaigrissement net, sans doute partiellement dû au changement de l'équilibre calorique des apports, car cet amaigrissement tend ensuite à s'estomper. L'éthylène-glycol produit lui aussi une perte de poids très significative. Dans l'association éthylène-glycol/citrate, les effets des deux facteurs ne sont pas additifs, c'est-à-dire que la perte de poids n'est pas plus forte qu'avec chacun des deux facteurs employés seuls. Le calcul fait apparaître une interaction négative très significative.

2° *Boisson* (tableau I). — La quantité de boisson absorbée en moyenne par Rat et par jour est sensiblement la même dans les quatre groupes. Cela tient sans doute à l'âge des animaux, car chez les jeunes rats, l'éthylène-glycol augmente généralement l'absorption de boisson.

3° *Poids des organes* (tableau II). — La rate, le cœur, les surrénales ne sont pas modifiés de manière significative, au moins lorsque l'on tient compte du poids corporel des animaux. Par contre, les reins présentent des variations particulièrement intéressantes : le citrate seul ne modifie pas leur poids, tandis que l'éthylène-glycol seul l'augmente nettement, comme il est habituel. Avec l'association citrate et éthylène-glycol, le poids des reins est sensiblement normal, ce qui indique une interaction négative significative entre citrate et éthylène-glycol. Au niveau du foie, s'observe également une action : éthylène-glycol et citrate diminuent tous deux le poids de cet organe, davantage dans le cas du citrate. La combinaison montre des effets additifs et pas d'interaction.

Tableau II. — *Interaction entre l'éthylène-glycol et le citrate à doses forcées chez le Rat. Poids des organes, urée et protides sériques.*

	Témoins	E.G.	Citrate	Citrate + E.G.	Différence entre groupes	E.G. seul	Citrate seul	Interaction
Poids rate (1) [mg]	593	561	579	597	N.S.	—	—	—
Poids cœur (mg)	919	977	974	965	N.S.	—	—	—
Poids reins (cg)	188	284	208	217	0,01	↗ 0,01	N.S.	(—) 0,05
Poids foie (cg)	1116	954	872	792	0,001	↘ 0,05	↘ 0,001	N.S.
Poids surrénales (mg)	48,5	48,5	45,0	41,8	N.S.	—	—	—
Urée (2) [cg/l]	44	288	47	47	0,001	0,001	N.S.	(—) 0,001
Protides sérum (g/l)	64,8	66,1	65,9	71,3	N.S.	—	—	—

(1) Sauf dans le cas des surrénales, les tests de signification sont calculés sur les poids ajustés sur ordinateur d'après la droite de régression poids d'organe/poids du corps calculée pour les témoins. Les poids moyens indiqués sont les valeurs ajustées.

(2) Tests de signification effectués après transformation logarithmique.

4° *Urée et protides du sérum* (tableau II). — Le taux de l'urée sanguine, nettement augmenté par l'E. G., seul reste normal dans le cas de l'association éthylène-gly-

blablement attribuable à une stimulation des enzymes du cycle de KREBS. En effet, le mélange de citrates utilisé ici modifie peu le *p*H urinaire chez le Rat, moins que le bicarbonate de sodium à doses égales, et moins que la solution d'EISENBERG, qui contient des citrates trisodique et tripotassique.

L'administration de doses fortes d'ion citrate à des rats mâles traités par de l'éthylène-glycol s'oppose à la création de la lithiase à l'oxalate de calcium habituellement obtenue sous l'effet du seul dialcool. De même est empêchée la néphrite tubulaire qui suit les dépôts cristallisés dans le rein. Cette expérimentation explique les résultats obtenus par l'emploi des citrates dans la thérapeutique des lithiases. Enfin, cette action spectaculaire permet de penser que l'emploi de citrates à doses fortes *per os* peut être préconisé dans le traitement de l'intoxication aiguë à l'éthylène-glycol.

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Annales pharmaceutiques françaises
1968, **25**, n° 9-10, pp. 599-602

Analyse des pommades.

VI. — *Etude de la composition du blanc de baleine par spectrométrie de masse,*

par

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RÉSUMÉ. — *Une analyse très fine d'un échantillon de blanc de baleine a été réalisée par chromatographie d'adsorption sur silice, suivie d'un examen des groupes chimiques ainsi séparés par spectrométrie de masse. Cette méthode a permis l'identification d'un nombre important de constituants non encore signalés jusqu'ici.*

Dans une récente publication [2] nous avions proposé une méthode d'analyse des cires en prenant comme exemple le blanc de baleine. Ce dernier avait été séparé en plusieurs fractions par chromatographie d'adsorption sur silice. La composition de chacune des fractions avait été étudiée par la chromatographie sur couches minces, la spectroscopie infra-rouge, la résonance magnétique nucléaire et la chromatographie en phase gazeuse. En dehors de traces d'hydrocarbures, trois groupes de constituants principaux avaient alors été isolés : des esters d'acides et d'alcools gras (fraction A), des triglycérides (fraction B) et un mélange d'acides et d'alcools libres accompagnés d'hydroxyesters (fraction C). Dans chacun de ces groupes, la chromatographie en phase gazeuse avait permis d'identifier les constituants indiqués dans le tableau I.

Le but du présent travail est d'approfondir l'étude de la composition du blanc de baleine en ayant recours à la spectrométrie de masse.

La spectrométrie de masse a été effectuée en utilisant un appareil Atlas CH$_4$ avec une source d'ions pour solides TO$_4$. Les spectres ont été enregistrés en utilisant une tension d'ionisation non seulement de 70 eV mais aussi de 11 eV de façon à

(*) Manuscrit reçu le 13 novembre 1967.

col/citrate, ce qui confirme au niveau du rein une forte interaction négative et s'accorde parfaitement avec les valeurs de poids des organes rapportées plus haut. Les protides sériques ne sont pas modifiés.

5° *Histologie.* — La lithiase rénale dont l'intensité est figurée par des +, selon une échelle décrite précédemment [2], est très importante chez les dix rats traités à l'éthylène-glycol seul : 1 rat a une lithiase à ++++, 2 rats à +++, 2 rats à ++, 5 rats à +. Elle est accompagnée d'une néphrite tubulaire chez tous les animaux intoxiqués. Par contre, chez les rats traités par du citrate associé à l'éthylène-glycol il y a une lithiase seulement chez 4 rats : un rat à +, trois autres ± ; chez deux de ces animaux lithiasiques les cristaux d'oxalate de calcium sont accompagnés d'une légère dilatation tubulaire et d'une légère atrophie épithéliale ; tandis que les deux autres, pas plus que les rats non lithiasiques, n'ont de modifications épithéliales des tubes. Les autres séries de rats n'ont pas de modification histologique rénale. Les autres organes sont dans toutes les séries sensiblement normaux.

Commentaires

Les citrates administrés à doses fortes par voie buccale permettent de s'opposer à la néphrite et à la précipitation d'oxalate de calcium produites par l'éthylène-glycol chez le Rat mâle. La cause et les conséquences de cette action méritent d'être évoquées.

1° *Métabolisme du calcium.* — Une des hypothèses envisagées pour comprendre comment les sels calciques des os sont dissous et passent dans le sang est que le citrate se formant dans le cycle de Krebs est l'agent chélateur en cause. Celui-ci interviendrait dans l'équilibre entre les éléments minéraux du liquide extracellulaire et les éléments minéraux de l'os, avec intervention de la parathormone qui agirait sur les os par l'intermédiaire de l'ion citrique pour libérer le calcium [8, 13]. Nous pouvons nous demander si l'augmentation des ions citrates dans l'organisme n'empêche pas la précipitation de l'oxalate sous forme de sel de calcium dans les reins par suite de la chélation du calcium. La calcémie peut, en effet, s'élever au cours de la néphrite à l'éthylène-glycol [3]. Plusieurs éléments s'opposent à cette hypothèse. Nous avons montré que la calcémie n'augmente dans la lithiase rénale à l'éthylène-glycol que lorsqu'il y a une forte intoxication par le dialcool seul ; en outre, nous avons pu dissocier la lithiase elle-même de l'hypercalcémie dans certaines séries d'expériences [21].

De plus, les Harrison [7] ont démontré que l'administration *per os* d'acide citrique n'augmentait pas le taux de la citrémie. Lichtwitz et ses collaborateurs [15] ont résumé l'état de la question dans les termes suivants : « ... l'acide citrique exogène traverse l'estomac sans modifications notables, mais il est détruit ultérieurement : soit dans sa traversée intestinale où il est métabolisé dans le cycle de Krebs des cellules de la paroi, soit dans le foie, car la veine sus-hépatique contient moins d'acide citrique que la veine porte... Il semble par contre, que cet acide citrique exogène joue un rôle important dans l'absorption intestinale du calcium », dans le sens d'une augmentation.

Enfin Elliot et ses collaborateurs [6] pensent que le rôle de l'acide citrique dans l'excrétion urinaire normale du calcium est peu important et pratiquement insignifiant dans la lithiase.

Cette première hypothèse devant être écartée, il reste à envisager celle d'une intervention dans le métabolisme du dialcool.

2° *Métabolisme de l'éthylène-glycol.* — L'intoxication lithiasique par l'éthylène-glycol est due essentiellement à son métabolisme conduisant à la formation d'acide oxalique. Nous avons émis précédemment l'hypothèse que la diminution de l'oxalose par les œstrogènes [19], par la phénacétine [21], par l'éthanol [4] pouvait s'expliquer par une compétition enzymatique où interviendraient comme coenzyme NAD et probablement NADP. Or, si la production du citrate endogène dépend surtout de NAD, son oxydation ultérieure nécessite NADP [9]. Nous pouvons donc imaginer que l'adjonction *per os* de doses fortes de citrates puisse, soit au niveau de l'intestin, soit au niveau du foie et probablement des deux, provoquer une forte augmentation des besoins en coenzymes au niveau du cycle de Krebs. De ce fait, l'éthylène-glycol, moins métabolisé, s'éliminerait tel quel et sans produire d'acide oxalique, donc de lithiase.

3° *Traitement des intoxications aiguës à l'éthylène-glycol.* — Nous avons suggéré, en cas d'une telle intoxication, d'essayer de bloquer les enzymes de dégradation du toxique en les captant par l'alcool éthylique, grâce à des perfusions d'éthanol dans du soluté glucosé par exemple [4]. Peterson [14] et ses collaborateurs ont augmenté la dose léthale 50 de l'éthylène-glycol chez le Rat par injection intrapéritonéale d'éthanol.

Wacker et ses collaborateurs [22], s'inspirant des résultats obtenus dans le traitement des intoxications par l'alcool méthylique, publient le résultat de leur traitement par l'éthanol d'intoxications à l'éthylène-glycol. En France, une démonstration des heureux résutats de cette méthode a été faite à l'hôpital Saint-Vincent-de-Paul, à Paris [10, 11]. Etant donné que les doses d'alcool éthylique nécessaires au traitement de l'intoxication par l'éthylène-glycol peuvent être importantes et qu'il faut évidemment éviter de provoquer un coma éthylique, nous suggérons d'essayer de compléter cette désintoxication par l'éthanol intraveineux par l'administration de citrates à fortes doses par voie buccale.

4° *Traitement des lithiases par les citrates.* — Cloarec et Rouffy [1] ont traité des malades avec une forte dose d'acide citrique et de citrates dans le but d'obtenir une chémolyse médicamenteuse de lithiases uriques. Ils ont pu alors constater un abaissement significatif des taux sanguins du cholestérol, des triglycérides, de l'urée et de l'acide urique. Lorsque Eisenberg, Connord et Howard ont préconisé en 1955, l'emploi d'une thérapeutique de ce genre dans le traitement de la lithiase, ils se basaient pour ce faire sur l'hypothèse d'une dissolution des calculs par modification du *p*H urinaire. Si l'on rapproche les travaux précédents de l'action hyperuricémiante de l'éthanol observée par Delbarre [5], il est permis de supposer que l'activité dans la lithiase des citrates à doses fortes est plus vraisem-

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Acta Facultatis Pharmaceuticae

Vol. XVII, 1969

Acidimetric Determination of Sodium Citrate in a Water-Acetone Medium

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A method has been worked out for the acidimetric determination of sodium citrate in a water-acetone medium. For the prupose of indicating the point of equivalence, use is made of a composite methyl orange - Nile blue A indicator; the suitability of utilizing this indicator for acidimetric determination has been confirmed through objective evaluation by means of additional tristimulation colorimetry. The results, which have been obtained in determining sodium citrate by the method worked out, are compared with the results studied by the application of other methods.

Sodium citrate, which represents an officinal (medicinal) preparation in the Czechoslovakian pharmacopaia[1], has found application in pharmaceutical practice in the capacity of an anticoagulating agent; it is employed, primarily, in the form of a 3.8% solution. It is also used successfully in the capacity of a solution stabilizer[2]. A determination of the substance, both in corformity with the pharmacopeia[1], as well as in agreement with the Czechoslovakian norm[3], is carried out through a conversion of the citrate, by means of incineration, to a carbonate, which is determined through dissolution in water by means of neutralized titration. The citrate is determined in the form of a 3.8% solution, in conformity with technical conditions[4], by conversion to a sulfate and by weighing the latter. However, in comparison with the abovementioned methods, it proves to be more suitable to determine sodium citrate with perchloric acid in a medium of anhydrous acetic acid, according to crystalline violet, in the capacity of an indicator[5]. In view

of the fact, that both the pharmacopeic method, as well as a determination in accordance with technical conditions, require much time, we directed our attention to the development of a method that would be suitable for the customary control both of an officinal substance, as well as of a 3.8% solution. At the same time, we made an attempt to eliminate the shortcomings of both methods and, simultaneously, to attain the same accuracy that is possessed by titration in an anhydrous medium.

Experimental Section

Instruments, chemicals, and indicators

For the purpose of measuring extinction curves, use is made of a "Spekel" spectrophotometer (the VEB C. Zeiss Company, Jena) and of a tray with a diameter of 1000 centimeters. The measurement of the pH, in the area of the point of equivalence, was carried out by means of an electron potentiometer, "Mul'tescope V" (Lab. pristroje -- Lab. instruments, scientific (national) patents), with the application of a glass electrode, SEV 2 (Labora, scientific patents) in the capacity of an indicator, and of a saturated calomel electrode, KALC 4 (Labora, scientific patents) in the capacity of a comparative one. The sodium citrate satisfied the requirements of the pharmacopeia,[1] 0.5 normal HCl (a factor established potentiometrically). Methyl orange (sodium salt), Spolek pre chemickou a hutni vyrobu (Society for Chemical and Metallurgical Production), scientific patents; Nile blue A -- sulfate, KERES (KEPEC), Federated German Republic. Masked indicator: 1 part of methyl orange (0.1% aqueous solution): 0.77 parts of Nile blue A (0.1% aqueous solution); prior to being used, the solutions are mixed in the cited ratio.

Results

Sodium citrate may be determined in a water-acetone medium by means of titration with a titration solution of hydrochloric acid, according to methyl

orange in the capacity of an indicator. The accuracy of the determination, however, depends on the suitable amount of acetone in the solution; this was manifested in employing a preliminary method,[6] that yielded noticeably lower results; we disclosed that the latter are due to the unsatisfactory amount of acetone. By studying the effect of the concentration of acetone on the content of sodium citrate, we established that an acidimetric determination of sodium citrate may be carried out with an outcome of correct results in a water-acetone medium, with a 36.5% concentration, by volume, of acetone in the titration exponent. Sodium citrate may be determined, in this case, by proceeding in such a manner that 680 - 690 milligrams of the substance are dissolved in 10 milliliters of water; to the solution are added 15 milliliters of acetone, and the citrate salt is determined acidimetrically. In developing the method, we encountered, in addition to this, difficulties that consisted in that the methyl orange indicator being employed had, in the water-acetone medium, a protracted color transfer. We attempted to eliminate this shortcoming by masking the methyl orange with a suitable pigment. With this purpose in mind, we at first plotted a titration curve for sodium citrate in the proposed water-acetone medium, and by means of it, we calculated the required titration exponent; the latter had a value of 2.3 pH (Fig. 1). After that, we masked the methyl orange with an indifferent pigment so that there should be formed, with the cited pT, a gray, intermediate tint. The gray color was obtained by masking the methyl orange with a Nile blue A pigment; the prepared masked indicator of methyl - Nile blue A possessed a marked and distinct color transfer from green through gray to violet.

((Graph on pg. 89 of text)) ((within this graph)): Fig. 1 Titration curve for sodium citrate in a water-acetone medium. (678.3 milligrams, 0.5 normal HCL, f is essentially equal to 1.0019) ((in the lower right-hand corner of the same graph)): (equivalent 0.5N HCl)/(mol. $C_6H_5O_7Na_3$).

The clarity in the color change of the masked indicator also had an effect on the form of the corresponding extinction curves (Fig. 2), which indicated both a sufficient distance of the extinction maxima from one another, in the case of various pH values, as well as a distinct isoasbestic point at 475 points; this represents a property that characterizes suitable masked indicators. At the same time, the masked indicator curves maintain, even in a water-acetone medium, the properties of curves for a simple indicator and pigment.

The fact that a masked indicator has more pronounced color transfer than methyl orange alone was objectively confirmed by the positions of their additional color points on a chromaticity diagram [7, 8, 9] (Fig.3). The color points of a masked indicator, which correspond to the graduated values of pH, are located on a straight line that passes very close to the C point, i.e., through the region of narrow color sectors; this attests to a clear change in color. On the contrary, the color points of methyl orange are located in the region of broader color sectors; consequently, methyl orange should possess weaker color contrasts than the masked indicator.

((two graphs on pg. 90 of text)) ((in the lower right-hand corner of the upper graph)): points; ((underneath the same graph)): Fig. 2 Extinction curves of the masked indicator, methyl orange - Nile blue A, for various pH values.

Curves:	1	2	3	4	5
pH:	2.0	2.4	2.6	2.8	3.2

masked indicator methyl orange - Nile blue A ------- ; methyl orange - - - - - ; Nile blue A - . - . - .

((in the upper left-hand corner of the lower graph)): 520 points; ((to the right of this graph)): Fig. 3 Chromaticity diagram, which represents the positions of additional color points of the masked indicator, depending on the pH. 1 -- methyl orange; 2 -- Nile blue A; 3 -- masked indicator methyl

orange - Nile blue A.

The method developed, with a convenient visual indication, used for the purpose of determining sodium citrate, yielded accurate results (Table 1) and was distinguished by a lower standard deviation and confidence interval[10] than the pharmacopeic method. At the same time, it is equally accurate as a method for determining sodium citrate in a medium of anhdrous acetic acid, with the application of both a crystalline violet, as well as a potentiometric indication (Table 1). The method developed is also suitable for determining sodium citrate in the form of a 3.8% solution (Table 1), where it is not possible to apply titration directly in an anhydrous medium.

Acidimetric determination of sodium citrate, in a water-acetone medium, is more advantageous than a pharmacopeic determination in that it reduces a high weighed portion, eliminating, simultaneously, the duration of the incineration. This is a very rapid and simple method.

Table 1

Determination of Sodium Citrate

NATRIUM CITRICUM, substance
By potentiometric titration, there was established a content of 99.59%*

Methods of Determination	Mean Result of Determination, in % **	+ s -	+ t -
ChsF - 2 (Czech.Pharmacopeia)	99.57	0.14	0.37
Anhydrous medium	99.40	0.06	0.16
Water-Acetone medium	99.42	0.07	0.18

NATRIUM CITRICUM, 3.8% solution

Content in conformity with TR (technical conditions), 3.65 - 3.95%

Determination in a Water-Acetone Medium

Batch	% Disclosed		Batch	% Disclosed	
020864	3.79	3.78	320866	3.77	3.76
100366	3.83	3.84	070167	3.81	3.80

* Average of 5 determinations — s is essentially equal to the standard deviation

** Average of 6 determinations — t is essentially equal to the confidence interval

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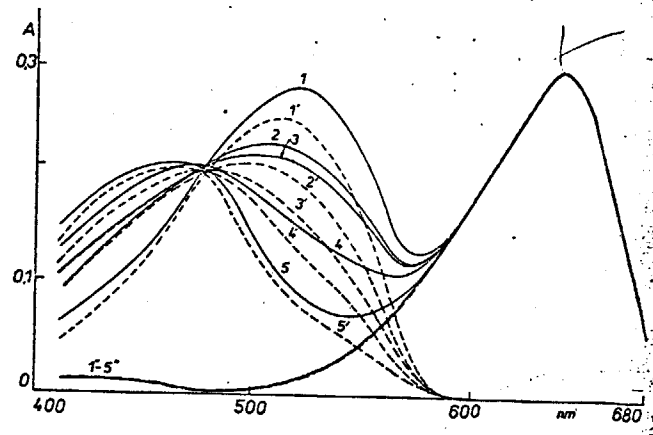

Рис. 2. Кривые экстинкции маскированного индикатора метиловый оранжевый — нильский синий А при различных значениях pH.

Кривые:	1	2	3	4	5
pH:	2,0	2,4	2,6	2,8	3,2

маскированный индикатор метилоранжевый — нильский синий А ———
метилоранжевый – – – – нильский синий А —.—.—

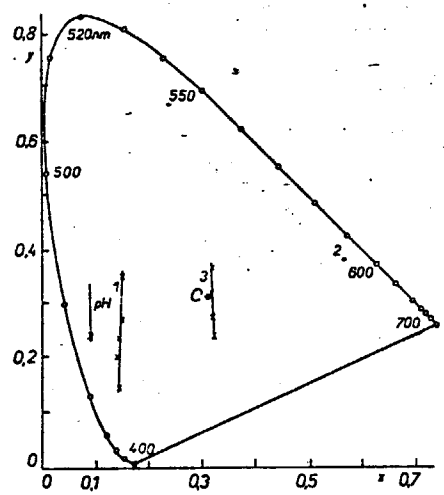

Рис. 3. Хроматицидная диаграмма, изображающая положения дополнительных цветных пунктов маскированного индикатора в зависимости от pH.
1 метилоранжевый 2 нильский синий А 3 маскированный индикатор метилоранжевый — нильский синий А

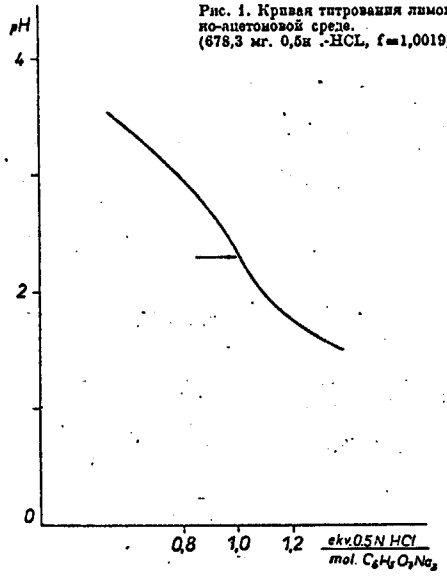

Рис. 1. Кривая титрования лимоннокислого натрия в водно-ацетоновой среде.
(678,3 мг. 0,5н .-HCL, f=1,0019)

Citrate #466

ACTA FACULTATIS PHARMACEUTICAE
TOM. XVII. 1969

АЦИДИМЕТРИЧЕСКОЕ ОПРЕДЕЛЕНИЕ ЛИМОННОКИСЛОГО НАТРИЯ В ВОДНОАЦЕТОНОВОЙ СРЕДЕ

МИЛАН ЗАГРАДНИЧЕК, ЯН ШУБЕРТ

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Разработан метод ацидиметрического определения лимоннокислого натрия в водно-ацетоновой среде. Для индикации точки эквивалентности применен смешанный индикатор метиловый оранжевый — нильский синий А; выгодность употребления этого индикатора для ацидиметрического определения подтверждена объективной оценкой с помощью дополнительной тристимуляционной колориметрии. Результаты, полученные при определении лимоннокислого натрия разработанным методом, сопоставлены с результатами, изученными с применением других методов.

Лимоннокислый натрий, являющийся официнальным в чехословацкой фармакопее[1], нашел применение в фармацевтической практике в качестве противосвертывающего средства; он употребляется преимущественно в виде 3,8 % раствора. Применяется с успехом также в качестве стабилизатора растворов[2]. Определение субстанции, как согласно фармакопее[1], так и в согласии с чехословацкой нормой[3], проводится переводом цитрата путем сжигания в карбонат, который определяется по растворении в воде с помощью нейтрализационного титрования. В виде 3,8 % раствора определяется цитрат, в согласии с техническим условием[4], переводом в сульфат и взвешиванием последнего. Однако по сравнению с упомянутыми методами оказывается более выгодным определение лимоннокислого натрия с хлорной кислотой в среде неводной уксусной кислоты по кристаллическому фиолетовому в качестве индикатора[5]. Ввиду того, что как фармакопейный метод, так и определение по техническому условию требуют много времени, мы направили внимание на разработку метода, подходящего для обычного контроля как официнальной субстанции, так и 3,8 % раствора. При этом мы стремились удалить недостатки обоих методов и

одновременно достигнуть той же точности, какой обладает титрование в неводной среде.

Экспериментальная часть

Приборы, химикаты, индикаторы

Для измерения кривых экстинкции применен спектрофотометр „Спекол" (фирма VEB C. Zeiss, Jena) и кюветы диаметром 1,000 см. Измерение pH в области точки эквивалентности проводилось с помощью электронного потенциометра „Мультоскоп V" (Lab. přístroje — Лаб. приборы, н. п.) с применением стеклянного электрода SEV 2 (Labora, н. п.) в качестве индикаторного и насыщенного каломельного электрода KALC 4 (Labora, н. п.) в качестве сравнительного. Лимоннокислый натрий удовлетворял требованиям фармакопеи.[1] 0,5н. HCl (фактор установлен потенциометрически). Метиловый оранжевый (натриевая соль), Spolek pro chemickou a hutní výrobu (Общество для химического и металлургического производства), н. п., нильский синий А — сульфат, KEPEC, ФРГ. Маскированный индикатор: 1 ч. метилоранжевого (0,1 % водный раствор): 0,77 ч. нильского синего А (0,1 % водный раствор); перед употреблением растворы смешиваются в приведенном соотношении.

Результаты

Лимоннокислый натрий можно определить в водно-ацетоновой среде путем титрования титровальным раствором хлористоводородной кислоты по метиловому оранжевому в качестве индикатора. Однако правильность определения зависит от удобного количества ацетона в растворе, что проявилось при применении первоначального метода,[6] дававшего заметно более низкие результаты; мы обнаружили, что последние обусловлены неудовлетворяющим количеством ацетона. Изучая влияние концентрации ацетона на содержание лимоннокислого натрия, мы установили, что ацидиметрическое определение лимоннокислого натрия можно проводить с получением правильных результатов в водно-ацетоновой среде при 36,5 % объемной концентрации ацетона в экспоненте титрования. Лимоннокислый натрий можно в этом случае определить, поступая так, что 680—690 мг вещества растворяется в 10 мл воды, к раствору прибавляется 15 мл ацетона и лимоннокислая соль определяется ацидиметрически. При разработке метода мы встретились кроме того с затруднением, заключающемся в том, что применяемый индикатор метиловый оранжевый имел в водно-ацетоновой среде растянутый переход окраски. Этот недостаток мы стремились удалить маскировкой метилоранжевого подходящим красителем. С этой целью мы сначала построили кривую титрования лимоннокислого натрия в предложенной водно-ацетоновой среда и с ее помощью вычислили нужный экспонент титрования; последний имел значение 2,3 pH (рис. 1). Затем мы маскировали метиловый оранжевый индифферентным красителем так, чтобы образовался при приведенном pT серый промежуточный оттенок. Серая окраска была получена маскировкой метилоранжевого красителем нильский синий А; приготовленный маскированный индикатор метилоранжевый — нильский синий А обладал резким и отчетливым переходом окраски от зеленой через серую до фиолетовой.

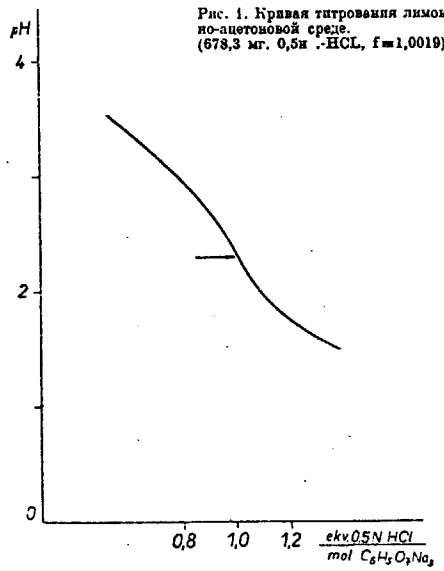
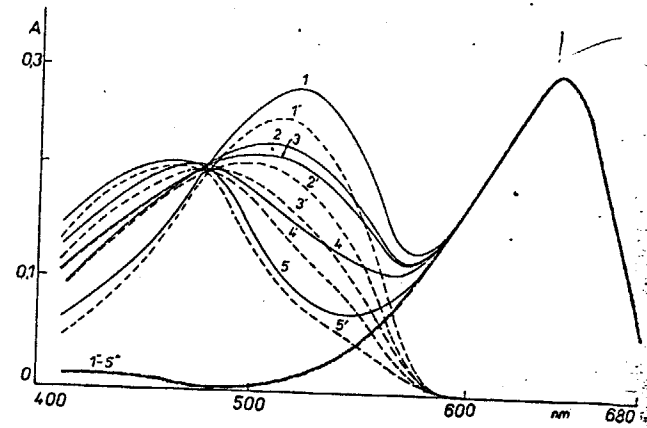


Рис. 1. Кривая титрования лимоннокислого натрия в водно-ацетоновой среде.
(678,3 мг. 0,5н .-HCL, f=1,0019)

Отчетливость изменения окраски маскированного индикатора сказалась и на форме соответствующих кривых экстинкции (рис. 2), которые показали как достаточное расстояние максимумов экстинкции друг от друга при различных значениях pH, так и отчетливую изосбестическую точку при 475 nm, что является свойством, характеризующим пригодные маскированные индикаторы. При этом сохраняют кривые маскированного индикатора и в водно-ацетоновой среде свойства кривых простого индикатора и красителя.

Факт, что маскированный индикатор имеет более резкий переход окраски, чем один метилоранжевый, был объективно подтвержден положениями их дополнительных цветных пунктов на хроматицидной диаграмме[7,8,9] (рис. 3). Цветные пункты маскированного индикатора, соответствующие градуированным значениям pH, находятся на прямой, которая проходит весьма недалеко от пункта C, т. е. через область узких цветных секторов, что свидетельствует об отчетливом изменении окраски. Наоборот цветные пункты метилового оранжевого находятся в области более широких цветных секторов; следовательно, метилоранжевый должен обладать более слабыми цветными контрастами, чем маскированный индикатор.

Разработанный метод с удобной визуальной индикацией, примененный



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Кривые:	1	2	3	4	5
pH:	2,0	2,4	2,6	2,8	3,2

маскированный индикатор метилоранжевый — нильский синий А ———
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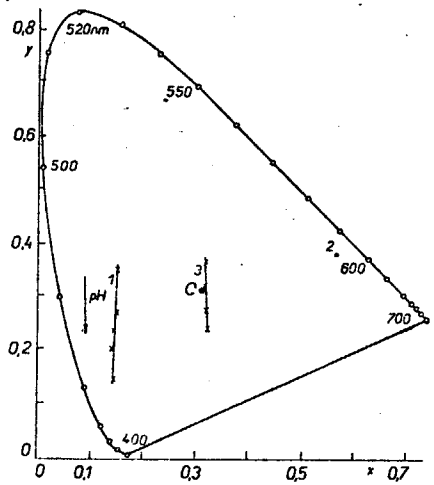

Рис. 3. Хроматицидная диаграмма, изображающая положения дополнительных цветных пунктов маскированного индикатора в зависимости от pH.
1 метилоранжевый 2 нильский синий А 3 маскированный индикатор метилоранжевый — нильский синий А

для определения лимоннокислого натрия, дал точные результаты (табл. 1) и отличался меньшим стандартным отклонением и доверительным интервалом[10], чем фармакопейный метод. При этом он одинаково точен как метод для определения лимоннокислого натрия в среде неводной уксусной кислоты с применением как кристаллического фиолетового, так и потенциометрической индикации (табл. 1). Разработанный метод подходит и для определения лимоннокислого натрия в виде 3,8 % раствора (табл. 1), где нельзя применить непосредственно титрование в неводной среде.

Ацидиметрическое определение лимоннокислого натрия в водно-ацетоновой среде выгоднее, чем фармакопейное определение в том, что понижает высокую навеску, удаляя одновременно длительность сжигания. Это весьма быстрый и простой метод.

Табл. 1

Определение лимоннокислого натрия

NATRIUM CITRICUM субстанция Потенциометрическим титрованием установлено содержание 99,59 %*			
Методы определения	Средний результат определения %**	+s–	+t–
ЧсФ — 2	99,57	0,14	0,37
Неводная среда	99,40	0,06	0,16
Водно-ацетоновая среда	99,42	0,07	0,18

NATRIUM CITRICUM раствор 3,8 % Содержание в согласии с ТР (техническое условие) 3,65—3,95 %				
Определение в водно-ацетоновой среде				
Партия	Обнаружен %		Партия	Обнаружен %	
020864	3,79	3,78	320866	3,77	3,76
100366	3,83	3,84	070167	3,81	3,80

*Среднее из 5 определений
**Среднее из 6 определений

s = стандартное отклонение
t = доверительный интервал

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a mixture of acetic anhydride (50 ml.) and conc. H_2SO_4 (1 ml.) and heated at 60–70° for 30 min. On cooling, *1:4:5:7-tetraacetoxy-2-methylanthraquinone* separated. Wt. 0·35 g. M.P. 221–226°. Two crystallizations from acetic anhydride raised the M.P. to 235°, not depressed on admixture with catenarin tetraacetate from *H. catenarium* (M.P. 235°). [Found: C, 60·94, 60·88; H, 3·80, 3·90; CH_3CO, 37·8, 38·05%. Calc. for $C_{22}H_{18}O_{10}$: C, 60·77; H, 3·99; $4CH_3CO$, 37·9%.]

Summary

1:4:5:7-Tetrahydroxy-2-methylanthraquinone has been synthesized. This compound was shown to be identical with natural catenarin, a metabolic product of different species of *Helminthosporium*.

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116. THE CITRIC ACID CONTENT OF ANIMAL TISSUES, WITH REFERENCE TO ITS OCCURRENCE IN BONE AND TUMOUR[1]

By FRANK DICKENS

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(*Received 1 September 1941*)

The distribution of citric acid in the animal body has not previously been systematically studied, for although Thunberg and his colleagues have made a careful examination of the citrate content of body fluids, that of the solid tissues has been largely neglected. This is all the more surprising, since Pucher *et al.* [1936] have described a sensitive colorimetric method which, unlike the enzymic one of Thunberg, is readily applicable to such material. Pucher *et al.* [1936] and Sherman *et al.* [1936] analysed only a few tissues by the colorimetric method (liver, kidney, abdominal and heart muscle of the dog), for which very low values were found (0·1–1·9 mg. citric acid/100 g. tissue). It is clear from their paper that they regarded these results as typical of the major tissues of the body, but this view is shown to be incorrect by the present work.

In contrast with these low results on solid tissues, it has long been known that several body fluids contain relatively large amounts of citrate. Thus milk contains enough for the crystallization of Ca citrate from evaporated milk, an occurrence which led to the first identification of citric acid as a normal constituent of the animal organism by Soxhlet & Henkel [1888]. Citric acid was not isolated from urine until many years later [Fasold, 1930], although it was shown to be a normal constituent by Amberg & McClure [1917], and in fact urine may also contain amounts of citric acid of similar order to that present in milk (20–120 mg./100 ml.; for literature see Östberg [1931], Scherstén [1936], Grönvall [1937], Sherman *et al.* [1936]). According to the Scandinavian workers, much less citric acid is present in cerebrospinal fluid, amniotic and follicular fluids, blood serum, aqueous humour, saliva and sweat, which contain in diminishing order about 5–0·1 mg./100 ml. Pucher *et al.* [1936] found similar low figures for blood and saliva. Finally there is the surprising observation of Scherstén [1929, 1936] that a high concentration of citric acid (up to 410 mg./100 ml.) is present in semen, and is particularly associated with the secretion of the seminal vesicles, which may contain as much as 633 mg./100 g. This brief outline of the present knowledge of citric acid distribution may be supplemented by the comprehensive reviews already quoted.

The present investigation is primarily the outcome of analyses of tumour tissue, a material of which the citric acid content has not previously been studied. The results showed that tumour frequently, though not invariably, contains a relatively high concentration of citric acid. However, certain normal organs

[1] The greater part of this work was reported to the Biochemical Society at Sheffield in Feb. 1940 [Dickens, 1940]. Its completion was delayed by difficulty in obtaining necessary replacements for the photometer used.

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such as skin, hair and embryonic tissue were found to have much higher amounts than those previously reported for solid tissues. In addition, the hard substance of bone has been shown to be a hitherto unsuspected store of citric acid, which may constitute as much as 1·6% of the dry, fat-extracted bone. It follows that the skeleton is the site of by far the greatest proportion of citric acid in the vertebrate, for it occurs in high concentration in the bones of all species yet investigated.

Experimental

Methods. Freshly excised tissues were used in all experiments except those on materials such as horse hair, ox bone or dried extracted bone. The animals received their last food 18 hr. before the experiment. Immediately after dissection and weighing, the tissue was ground with sand and extracted three times with 10% trichloroacetic acid. When bone was analysed it was first chopped or pulverized, then ground with sand and digested, usually with more concentrated (40%) trichloroacetic acid at about 30°, until the hard substance had dissolved. The solid residue was then re-extracted with 10% trichloroacetic acid as above. After boiling down the combined extracts with H_2SO_4 (final conc. *ca.* *N*) and cooling, the precipitated $CaSO_4$ was removed by centrifuging.

The citric acid was estimated by the colorimetric pentabromoacetone method of Pucher *et al.* [1936], with dioxan [Johnson, 1939] instead of pyridine as the final diluent. This method of analysis is the best available, but there are some sources of error which need special attention. The fresh, filtered solution of Na_2S must be prepared immediately before use. If an aliquot part of the trichloroacetic filtrate after boiling with H_2SO_4 is taken, it is important that after dilution further H_2SO_4 is added to make about *N* concentration before the oxidation. The use of $FeSO_4$ to decolorize the $KMnO_4$ was not found consistently reliable [cf. Thunberg, 1941, 1] owing to the serious effect of even minute traces of residual Fe after washing, and instead the method of Pucher *et al.* [1934] using H_2O_2 at 6° instead of $FeSO_4$ has been found to be preferable. The addition of H_2O_2 must be carried out with all the care of a titration, a slight excess leading to rapid destruction of pentabromoacetone. Some specimens of light petroleum were unsatisfactory, as Pucher *et al.* [1936] state. Instead of using water in the blank for colorimetry, the mixture of Na_2S and dioxan was employed. By this method, accurate estimations are obtained between 0·1 and 1·0 mg. citric acid: the specificity is discussed by Pucher *et al.* [1936] and is referred to in the discussion of the present paper.

Throughout the results, citric acid is always expressed as the anhydrous substance and with the exception of dried bone, tissue weights are wet weights.

The citric acid content of tumour tissue

Table 1 shows the citric acid contents of some typical tumours. It will be seen that the animal tumours used all had large amounts, from 12 to 18 mg./100 g. These tumours, of mouse, rat and rabbit, were fairly large and varying degrees of necrosis were present. In a suitable tumour, which could be dissected into mainly necrotic and mainly non-necrotic portions, the citric acid was estimated separately in the two. The results (Table 1) show that the non-necrotic part of this Crocker mouse sarcoma contains nearly twice the concentration of the necrotic tissue. The high citric acid content is therefore a property of the tumour tissue itself, and not the result of a change, such as calcification, accompanying the necrosis. In one experiment the liver of a rat bearing a Guérin tumour was analysed simultaneously with the tumour; the liver had less than 1/5 of the

Table 1. *The citric acid content of tumour tissue*

Species	Tumour	Citric acid mg./100 g.
Rabbit	Brown-Pearce carcinoma	13·6
Rat	Walker 256 carcinoma	16·6
,,	Walker 256 carcinoma	18·4
,,	Guérin tumour	16·8
,,	Guérin (liver of same rat)	(3·0)
Mouse	Crocker sarcoma 180	14·2
,,	Crocker sarcoma 180	14·3
,,	Crocker sarcoma 180 (mainly non-necrotic)	12·3
,,	Crocker sarcoma 180 (mainly necrotic)	(7·7)
Man	Malignant dysgerminoma (seminoma) of testis	9·9
,,	Fibroadenoma of uterus	5·0
,,	Carcinoma of stomach	2·7
,,	Adjacent gastric mucosa	(2·0)
,,	Carcinoma of vulva	8·8
,,	Carcinoma of vulva	7·0
,,	Leucoplakia of vulva	8·2
,,	(Adjacent normal skin)	(5·3)

Bracketed values refer to same animal or same case.

citrate content of the tumour (Table 1). Among human tumours the results were more variable; although high values were found for the carcinoma of the vulva and seminoma, the citric acid content of the carcinoma of stomach examined was only slightly higher than that of the adjacent gastric mucosa. In one case of carcinoma of the vulva, it was possible to compare the carcinoma with the nearby leucoplakia (which may be considered as precancerous) and adjoining skin which were excised together with the carcinoma; it will be noted that the citric acid content was greater in both the precancerous and malignant tissues than in the normal skin. The fairly high value obtained for the latter may be compared with the still higher values found below for animal skin.

Comparison of the total citric acid of the tumour with that of the remainder of the animal. Since low values have hitherto been reported for the total amounts of citric acid in animal organs, it appeared that analysis might show the greater part of the citric acid of a tumour-bearing animal to be contained in the tumour. This expectation was not, however, realized. A mouse bearing a Crocker sarcoma of weight about equal to that of the animal itself was selected, and the tumour and mouse were separately analysed (Table 2). In spite of the high content of

Table 2. *Analysis of a mouse tumour and of remainder of mouse*

	Total wt. g.	Citric acid mg.	Citric content mg./100 g.
Crocker sarcoma 180	20·8	2·95	14·2
Remainder of mouse	25·2	3·62	14·4

citric acid in the tumour (14·2 mg./100 g.), this contained only 45% of the total citric acid present in mouse and tumour together. It followed that some rich source of citrate was present in the tumour-free mouse, which brought up the citrate content of the whole animal to the high level of 14·4 mg./100 g.

The possibility that the intestinal contents were responsible was excluded by analysis of another mouse receiving the same diet. This animal weighed 32·1 g. and contained a total amount of 4·17 mg. citric acid, towards which the stomach, intestines, bladder and their contents (4·1 g.) contributed only 0·253 mg.

Complete analysis of the normal animal

In an endeavour to clear up this anomaly, the analyses shown in Table 3 were made. It will be noticed that the citric acid contents reported for the rabbit, guinea-pig and mouse are on the whole somewhat higher than those of

Table 3. *Citric acid content of tissues of adult animals*

Species	Tissue	Citric acid content mg./100 g.
Rabbit	Liver	2·8
	Skeletal muscle	2·5
	Kidney	6·0
	Whole brain	4·6
Guinea-pig	Liver	1·6
	Kidney	3·9
	Whole brain	3·8
	Testis	11·5
Mouse	Skin, fur mostly removed	12·2
	Fur	133
	Seminal vesicles	128
Mouse	Fur, total citric acid	114
	Fur, hot water extract	83
Mouse	Fur	93
	Skin, fur mostly removed	9·7
Horse	Hair, unwashed	27·7*
	Hair (commercial 'washed horse hair')	21·1
Man	Sebaceous cyst from hand	11·7
	Sebaceous cyst, mainly cyst wall, with only traces of adhering contents	5·0
Mouse	Skeleton, with eyes and closely adherent connective tissue	46†
	Muscle	2·8
	Skin, fur and tail sheath	8·6
	Seminal vesicles, testes and prostate	119
	Viscera and brain	4·6

* 16·2 mg./100 g. were hot water soluble.

† As it was not possible to remove a good deal of adherent tissue, this value is too low for the skeleton alone.

dog kidney, muscle and liver (0·1–1·9 mg./100 g.) reported by Pucher *et al.* [1936]. In agreement with the results of Scherstén [1936] for other species, a high content of citric acid is present in the seminal vesicles and total male gonads of the mouse, and in the testes of the guinea-pig. Low concentrations are present in skeletal muscle of rabbit and mouse (2·5, 2·8 mg./100 g.) and in liver of rabbit and guinea-pig (2·8, 1·6 mg./100 g.), while the brains of these two species have rather larger amounts (4·6, 3·8 mg./100 g.). Kidney tissue of rabbit and guinea-pig contains considerably more (6·0, 3·9 mg./100 g.) than that of the dog (1·9, 1·2 mg./100 g. [Pucher *et al.* 1936]). The skin and hair are fairly rich sources of citric acid (cf. also human abdominal skin, Table 1). The very high content of horse hair and particularly of mouse fur was very surprising; contamination by urine or semen may be excluded as the source of this citric acid, since it occurred in both male and female mice to the same extent, and care was taken to avoid contamination. It is of interest that a large proportion of the citric acid of hair is extractable by water (200 vol., boiled with hair for 5 min., extracts 58% of the total citrate from horse hair and 73% from mouse fur). It seemed possible that the citric acid arose from secretion of glands in the skin, and since mice are stated not to sweat on the furry parts (in any case sweat has

only 0·1–0·2 mg. citrate/100 ml. [Scherstén, 1936]), it was thought that the sebaceous glands might be the source. This hypothesis could hardly be tested in the mouse, but analysis of a sebaceous cyst from the human hand showed the fairly high citric acid content of 11·7 mg./100 g., towards which the cyst wall contributed only a minor part (Table 3). No doubt part of the high citric acid content of the skin may be attributed to a similar origin.

In spite of its high concentration, the citric acid in the fur and skin of a mouse comprises only a small fraction (about 10%) of the total in the animal. Similar considerations apply to the seminal vesicles, which in the adult mouse might add a further 5% to the total. Even excluding these known rich sources of citric acid, there is an overall concentration in the residue of the animal of over 10 mg./100 g.

The source of the greater part of this citric acid was revealed by the complete analysis of a whole mouse after dissection (Table 4). It will be seen that about

Table 4. *Analysis of a dissected mouse*

Male adult, fed 18 hr. before, live wt. 31·2 g.

Tissues	Tissue wt. g.	Citric acid mg.	Citric acid % of total in mouse
Skeleton, with closely adherent connective tissue and eyes	8·2	3·75	69
Muscle, with dissected connective tissue and a little fat	6·8	0·19	3·5
Outer skin, including skin of tail, and fur	4·3	0·37	6·8
Seminal vesicles, testes and prostate	0·6	0·71	13·1
Liver, spleen, brain, heart, lungs, kidneys and other viscera	9·0	0·41	7·5
Total	28·9	5·43	99·9

70% of the total citric acid in the mouse is contained in the skeleton, and this provides the explanation of the remarkably high citric acid content of the whole animal.

Bone, cartilage and marrow

Bone from the foreleg of an ox was obtained from the butcher, and the marrow removed. Great difficulty was experienced in crushing the hard bone, and in consequence its solution in trichloroacetic acid was incomplete; the citric acid content found is therefore probably low. Nevertheless the result showed that while the hard substance contained at least 272 mg./100 g., the red marrow had only 41 mg./100 g. Hence the greater part of the citric acid is contained in the hard bone. A similar experiment with bone and cartilage from a kitten (Table 5) showed that the content of citric acid in the cartilage is less than 10% of that in the bony substance, including marrow. In this and in the following experiments with bone, better solution of the hard material was obtained by taking 1–2 g. quantities of fresh substance, chopped when possible, and grinding with sand, 5 g. trichloroacetic acid and 1 ml. water (or, in the case of dried, extracted bone, 0·3 g. finely powdered material with 5 g. trichloroacetic acid and 10 ml. water) and keeping overnight.

I am indebted to Mrs C. M. Burns for the specimens of bone from specially treated animals, and for kindly supplying the analyses for Ca, P and CO_2, which are included with the citric acid determinations in Table 5. For technical details of preparation and analysis reference may be made to Burns & Henderson [1935].

Table 5. *Bone analyses*

	Citric acid mg./100 g.
Fresh bone, marrow and cartilage	
Ox	
Bone of foreleg	272
Red marrow from this bone	41
Kitten (3 days old)	
Bone, including marrow, from ribs and legs	373
Cartilage, from ribs and leg joints	33
'Steamed bone meal' (ox and sheep)	1420
Dried, alcohol-extracted, powdered bone	
Pup A. Normal	1310
Pup B. After prolonged administration of parathyroid hormone	1660
Kitten C. Normal	644
Kitten D. Rachitic; low Ca diet	345

Ca and P analyses

	Blood Ca mg./100 ml.	Bone analysis (%) Ca	P	CO_2
Pup A	11·2	21·4	10·8	2·42
Pup B	13·8	20·6	10·3	2·59
Kitten C	N.D.	22·7	11·3	2·78
Kitten D	N.D.	20·0	9·59	2·43

Bone cortex was taken from the middle of the shaft, and samples are thus directly comparable in all experiments: after drying and extraction the bone was reduced to a fine powder and was dried to constant weight. The two pups, A and B (Table 5), were fed on identical diets which consisted largely of milk and porridge; B received massive and prolonged dosage with parathyroid extract. The effect of diet was studied in kittens C and D; C was fed on the usual mixed domestic diet while D received a similar diet but without milk or bones. This diet was clearly deficient in Ca, for whereas the bones of animal C although thin were not rachitic, those of D were definitely rachitic, being typically bent and having large epiphyses. The result of this experiment is discussed later in this paper, but attention may here be drawn to the increase in citrate in the bone of pup B which received parathyroid extract, and the fall in citric acid in kitten D which was on a diet deficient in Ca, and was rachitic. The great variation between the two species is also noteworthy.

The composition of steamed bone meal

The great hardness of fresh bone made it necessary, for the purpose of isolating citric acid, to find a more tractable material, which was readily available in 'steamed bone meal'. This is prepared commercially from coarsely pulverized bone, mainly of ox and sheep, which after mechanical separation from skin, hair, etc. is extracted with light petroleum and with steam, and is finally dried and reduced to a fine powder. The specimen used contained 4·20% moisture (6 hr. at 105°), 2·0% ether-extractable (mainly fat), 0·82% protein-N (*ca.* 5% protein); of the total meal 7·8% was insoluble in dilute HCl. The acid-soluble part (88·0% of the original meal, dry wt.) contained 30·3% Ca, 14·0% inorganic P and 16·05% total P, all calc. on the original wt. of meal.

The citric acid content (Table 5) was 1·42% and the CO_2 content 2·06%, both calc. on the original meal (i.e. 1·61 and 2·34% respectively, calc. on dry, fat- and protein-free, acid-soluble meal). Comparison with a typical analysis of

the 'inorganic' phase of human bone (Table 6) shows that the 'inorganic' part of the sample of bone meal used in these experiments has sustained some loss of CO_2 and Ca, but on the whole closely resembles the typical bone analysis in its mineral composition. This is regarded as evidence of the suitability of this material for comparison with natural bone.

Table 6. *Comparison of the compositions of normal bone ash and of 'steamed bone meal'*

	Bone meal, % of dry, fat- and protein-free wt.	Inorganic residue of bone (human) [Gabriel, 1894] %
CaO	48·2	51·31
P_2O_5 (inorganic)	36·5	—
(total)	41·8	36·65
CO_2	2·34	5·86
Citric acid	1·61	—
Minor constituents	—	5·92*
	93·95	99·74

* Water, 3·78; MgO, 0·77; K_2O, 0·32; Na_2O, 1·04; Cl, 0·01%.

The solubility of citric acid present in bone meal. 1 g. bone meal was shaken for 2 hr. with 100 ml. distilled water at room temperature and filtered. The clear filtrate (95 ml.) contained 0·629 mg. citric acid, or 5% of that present. Boiling with 200 ml. water extracted from the residue only a further 1·24 mg. (10% of the total). Since the solubility of $Ca_3Cit._2,4H_2O$ (Beilstein, III, 564) is 85 mg./100 ml. at 18°, equivalent to 57 mg. citric acid, it is evident that the citric acid in bone meal, if present as the Ca salt, must be in some form not readily extractable by water. The presence of appreciable amounts of free citric acid, which is in any case improbable, or of the freely soluble alkali salts seems to be excluded by this experiment.

The isolation of citric acid from bone meal

On account of the great preponderance of $Ca_3(PO_4)_2$ the isolation of a pure specimen of citric acid was accompanied by much loss; but the isolation in good yield of pentabromoacetone, as described later, appears to make further work on these extractions of citric acid hardly necessary. Two methods were used, of which the first is more laborious and more wasteful, but yields a product which is readily obtained quite pure.

Isolation by fractional precipitation. 1 kg. bone meal was extracted three times at 100° with 2·5 l. of 2*N* HCl. The filtered extract was made alkaline with NH_3 and the precipitate collected. The precipitate contained 93% and the filtrate only 5% of the citric acid initially present. The moist precipitate was thoroughly stirred with 1 l. water and 0·67 l. 18*N* H_2SO_4. After adding 1·7 l. alcohol and filtering, the filtrate was evaporated at 30° to remove alcohol; magnesium acetate (0·5 kg.) was added followed by ammonia to make alkaline. The precipitate of phosphate was removed, and to the Ca- and PO_4-free solution were added 100 g. Ba acetate and 1·9 l. alcohol. The precipitate of Ba salts was collected and washed with 70% alcohol. Three extractions of the precipitate with a mixture of 90 ml. alcohol and 10 ml. conc. HCl were combined and concentrated almost to dryness (with considerable accidental loss at this stage). The residual syrup was treated with 50 ml. 10% $CaCl_2$ and the boiling solution made alkaline with NH_3. The Ca salt, after washing and drying, weighed 2·15 g.; it

was freed from Ca by the calculated amount of N H_2SO_4 in 50% alcohol. The syrup remaining after evaporation on the water bath was dried; yield 0·55 g. It was recrystallized, with a trace of animal charcoal, from ether by refluxing with 60 ml. for 6 hr., evaporating the greater part of the ether, and keeping in a stoppered flask. The sparkling white crystals were washed twice with ether. Yield 0·325 g., M.P. (slow heating) 153·0° corr., mixed M.P. 153·5° corr., M.P. of authentic anhydrous citric acid 153·5° corr.

Direct extraction after acidification. 95 g. bone meal were extracted with ether for 3 hr. in a Soxhlet apparatus to remove fat. The dried residue was cooled in ice and well stirred with 95 ml. 7 N H_2SO_4. Three ether extractions of this paste, each of 24 hr., were combined. The yields of citric acid in each, by analysis, were 31, 26 and 3·0% of the total present. The extract was shaken with $NaHCO_3$ and the alkaline extract acidified with HCl; CO_2 and dissolved ether were removed on the water bath, and after boiling with animal charcoal the citrate was precipitated by addition to the boiling solution of 35 ml. 25% $CaCl_2$ and NH_3 to make alkaline. The precipitate of Ca salt was collected, washed twice with boiling water and dried. Yield 1·778 g. Ca salt containing by analysis 0·749 g. citric acid, or 60·2% of that present in the original meal. The Ca salt was ground with H_2SO_4 (16·6 ml. N) in 50% alcohol, filtered, and the precipitate re-extracted with 20 ml. 50% alcohol. The combined extracts were evaporated; the residue was dried in the oven, dissolved in a little alcohol (later experience showed this to be undesirable) and refluxed with ether. After removal of some insoluble material, the evaporated ether solution largely solidified on long keeping in a vacuum desiccator to a yellow crystalline residue (0·743 g.) which, however, still contained some alcohol. It was redissolved, with a trace of animal charcoal, in 20 ml. ether, filtered, evaporated and dried. Yield of nearly pure citric acid 0·502 g. = 40% of that calculated from analysis of original bone meal. The pale yellow crystals after one recrystallization from anhydrous ether yielded pure white citric acid, M.P. 152–153° corr.

Preparation of pentabromoacetone from bone meal

The following is based upon the preparative methods described by Wöhlk [1902] and Grönvall [1937]. 50 g. bone meal, containing by analysis 0·620 g. citric acid, were ground with a solution of 170 g. trichloroacetic acid in 100 ml. warm water. Rapid solution of all but a few coarse bony particles occurred, and after 30 min. the liquid was separated by filtration and centrifuging. To the filtrate cooled in ice 110 ml. 18 N H_2SO_4 were added (temp. $<15°$). The precipitate of $CaSO_4$ was well washed with water and the combined filtrate and washings (700 ml.) were evaporated over a flame to 500 ml.; two lots of 750 ml. water were added, boiling down to 500 ml. again after each addition, to destroy trichloroacetic acid. After keeping overnight in the ice-box, the clear solution was filtered from $CaSO_4$ and some fatty material.

5 g. KBr were added, and the solution warmed to 30° while 50 ml. 5% $KMnO_4$ were added. During 30 min. at 30° three lots of 10 ml. 5% $KMnO_4$ were added at intervals; the mixture was then decolorized with 30% $FeSO_4$, when a white crystalline precipitate appeared immediately, and more separated overnight at 0°. Yield 1·165 g. of nearly pure pentabromoacetone, M.P. 68–70° (equivalent to 0·495 g. citric acid or 80% of that found by analysis of the original bone meal). After one recrystallization from a little 50% alcohol, brilliant white crystals, M.P. 73°, mixed M.P. 73°, M.P. of authentic pentabromoacetone 73°, were obtained.

Citric acid in the embryo

The embryo is of particular interest in connexion with citric acid, because the mainly carbohydrate metabolism of the young embryo [Dickens & Šimer, 1930] and the later extensive calcification are both processes in which citric acid may be expected to play a part. Table 7 gives some preliminary results on this

Table 7. *Citric acid content of the embryo*

Species	Age	Average wt. of one embryo g.	No. of embryos for analysis	Citric acid mg. per embryo	Citric acid mg./100 g. embryo
Mouse	—	0·9	9	0·112	12·4
Mouse	New-born—a few hr. old	1·2	7	0·194	16·2
Chick	3½ days	0·037	6	(0·012)*	(31·0)*
	5 ,,	0·217	6	0·059	27·4
	8 ,,	1·263	2	0·168	13·3
	12 ,,	5·60	1	0·740	13·2
	21 ,,	48·60	1	4·56	9·4

* At lower limit of accuracy of the method.

material, from which it appears that an unusually high content of citric acid is indeed a feature of embryonic tissue. This is particularly marked in the earliest stage which can be readily investigated with a method of the sensitivity of the present one (3½- to 5-day chick); thereafter up to the 12th day the citric acid falls to a level comparable with that of the more advanced mouse embryo, the new-born mouse, and indeed the adult mouse (cf. Table 4). In comparing these results with the adult values, however, it must be remembered that 70% of the citric acid of the adult mouse is contained in the skeleton. The determination of the distribution of citric acid in the organs of the embryo should therefore be particularly informative. This is also a necessary step before any useful comparison with the citric acid content of tumours, that is of normal and pathological growth, can be made.

Discussion

The main result of this investigation is the discovery of citric acid as a major constituent of normal bone. The preliminary estimations [Dickens, 1940] have now been confirmed for the bones of several animals by Thunberg [1941, 1], who found by the colorimetric method that the bone substance of all species studied (horse, ox, sheep, pig and also the hen and 5 species of fishes) contained citric acid amounting to '1 bis einige Prozent der Trockensubstanz'. He also found about 0·5% of citric acid in the teeth of the cat, ox and pig. These results, together with those on the bones of the mouse, ox, kitten and puppy and on mixed bone meal which are contained in the present paper, show that the presence of a high concentration of citric acid is a very widespread, probably general, property of bone.

That the substance estimated is really citric acid, and not some unknown constituent giving a similar colorimetric reaction, is shown first by the isolation of pure citric acid from bone meal and secondly by the isolation of pure pentabromoacetone in 80% of the yield calculated from colorimetric analysis. This latter proof may reasonably be taken to mean that practically the whole of the substance is citric acid, for the isolation would not be expected to be entirely quantitative, and the formation of pentabromoacetone is such an unusual reaction, depending as it does on the intermediate formation of the enolic form of αα′-acetonedicarboxylic acid, that it is very highly specific for citric acid [cp.

Grönvall, 1937, pp. 62 *seq.* for literature]. Tartaric, succinic, malic, lactic, oxalic, pyruvic, camphoglycuronic, uric, tricarballylic, *trans*aconitic, itaconic, citraconic, and mesaconic acids are stated not to interfere, nor do glycerol, sucrose, glucose, $CaSO_4$ or $Ca_3(PO_4)_2$. Pucher *et al.* [1936] add to this list acetone, glycogen, acid haematin, creatine, creatinine, cholesterol, amytal, urea, taurine, acid digest of casein, allantoin: hippuric, maleic and fumaric acids: they found, however, that β-hydroxybutyric gave about 1/1000–1/200 and the ethyl ester of acetone-dicarboxylic acid about 1/110 of the colour given by citric acid. *cis*Aconitic acid gives no colour [Johnson, 1939]. From these data it must be concluded that there is no known physiologically occurring substance other than citric acid which can be held responsible for the pentabromoacetone prepared from bone.

The fact that so large a quantity of a familiar substance has hitherto escaped detection by bone analysis is surprising, but it is upon bone ash that the most accurate and complete analyses have been made, and in these the citrate would presumably appear as an addition to the carbonate fraction. It has long been recognized that the nature of the organic combination of bone cations is an almost unknown field, and a systematic study of bone for the presence of other physiologically important organic acids would now appear profitable.

Since the citric acid of bone meal is insoluble in alcohol and ether and is only slightly extracted by water, it is assumed that it may be present in combination with calcium, though this combination may be in the form of a complex ion, such as citrates are known to form with Ca [cf. Hastings *et al.* 1934]. The nature of its distribution throughout the bone, and its possible entry into the mixed crystal structure of bone, are questions for future investigations.

The mere presence of the highest concentration of citrate in that tissue which is richest in calcium, however, seems to provide by far the most striking evidence for a participation of citric acid in calcium metabolism. The idea of a citrate-like substance acting as a solvent for bone salts is a very old one [cf. Klinke, 1928], and Greenwald [1926] in his studies of parathyroid activity went so far as to postulate the formation of such a substance directly or indirectly by the parathyroid gland. He actually tested for citrate, presumably in the blood, but with negative results [Greenwald, 1926]. Thunberg and his associates have continually referred to the association of citric acid with calcium in the body, a striking example being the high content of citrate in semen, which has some 50 mg./100 ml. of Ca, about 5 times the concentration in plasma, and also has a high concentration of inorganic phosphate, without the precipitation of Ca occurring [Scherstén, 1936]. The high citric acid contents of skin and embryo, reported in the present paper, may be considered as strengthening this connexion, for skin has on a dry weight basis 'considerably more Ca and less Mg and P than in most other soft tissues' [Schmidt & Greenberg, 1935]; the case of embryo is discussed below. Recently, Thunberg [1941, 1, 2] has demonstrated the presence of 0·08–0·35 % of citric acid in the shell of eggs from many species of birds.

Since it is now well recognized that bone substance is continually in a state of vigorous metabolic exchange with body fluids [cp. Hevesy *et al.* 1940] it may be expected that the citrate of bone will accompany the other bone salts during their mobilization and deposition. Thus the stores of citrate in the bone will probably be available for the metabolic requirements of body tissues in general, whether catalytically in carbohydrate oxidation [Krebs & Johnson, 1937] or as solvent for Ca salts in Ca metabolism. In particular, it seems probable that the presence of citrate in the immediate vicinity of calcifying or decalcifying bone might, by its well-known solvent action, play an important part in preventing premature precipitation of bone salts in this region.

The present evidence is certainly far too meagre to decide if this mechanism is really in operation or not. Some tentative support for a participation of bone citrate in calcification may, however, be claimed from the analyses of Table 5. On the basis of these individual results, it appears that the bone citrate is considerably affected by both hormonal and dietary conditions. In fact, it is much more affected than the other salts of bone, as is clearly shown by the recalculation of the data of Table 5 in terms of the molecular proportions of the three main constituents (Table 8). On the basis of these figures, parathyroid administration,

Table 8. *Molar ratios in dried extracted bone*

	Ratio of atoms (or mol.) of				Estimated molecular proportions* of		
	Ca	P	CO_2	Citric acid	$Ca_3(PO_4)_2$	$CaCO_3$	$Ca_3Cit._2$
Pup A, normal	78·3	50·8	8·04	1	100	33	2·1
Pup B, parathyroid	59·5	38·4	6·80	1	100	38	2·7
Kitten C, normal	198·5	168·2	18·8	1	100	32	0·9
Kitten D, rickets	278·2	172	30·8	1	100	37	0·6
(Dried bone meal	116	69·4	7·06	1	100	20	1·4)

* Approximate only, based on the assumption that all the P is present as $Ca_3(PO_4)_2$, all CO_2 as $CaCO_3$ and all citric acid as $Ca_3Cit._2$.

tending to hypercalcaemia, increases the relative molar proportions of citrate to $Ca_3(PO_4)_2$ and, to a less extent, to $CaCO_3$. On the other hand, a diet deficient in calcium has the opposite effect: the citrate is diminished by some 30 %. The weakness of this evidence needs no emphasis; the normal variation has not been taken into account, and in the second experiment the diet deficient in Ca, from which milk and bones were omitted, was almost certainly also low in citric acid content. It shows, however, that the variations in citrate do greatly exceed those of the other salts; further experiments are being undertaken by Mrs Burns and it is hoped that these may provide more definite evidence. In this connexion, attention may be drawn to the calcifying action of citric acid and of citrates when added to a rachitic diet [cf. Hathaway & Meyer, 1939; Day, 1940].

The embryo. It is instructive to compare the data of Table 7 for the citric acid content of the chick embryo with those of Mankin [1929] on the Ca content (Table 9). Calculation of the ratio of equivalents of Ca to those of citric acid

Table 9. *Relationship of Ca to citric acid in the embryo chick*

Age in days	Average wt. g.	Ca, mg./100 g. embryo wet wt. [Mankin, 1929]	Citric acid mg./100 g.	Equiv. Ca / Equiv. Cit.
3½	0·037	—	31·0	3·3 : 1
4	0·144	32	—	
5	0·217	—	27·4	3·9 : 1
5	0·183	33	—	
8	1·263	—	13·3	5·8 : 1
8	1·010	24	—	
12	5·6	—	13·2	18·8 : 1
12	5·82	77·5	—	
21	—	381	9·4	130 : 1

(column 5) reveals the remarkable fact that in the earliest stages capable of investigation by the present method (4–5 days), the citrate present in the whole embryo is sufficient to combine with almost 1/3 of the total Ca. Thereafter, the total Ca increases much more rapidly than the citric acid.

The presence of so high a concentration of citrate relative to Ca, may, if it proves to be a general feature of embryonic metabolism, be of great importance in securing an adequate supply of readily mobilizable Ca for the use of the very young embryo.

It seemed probable that the shell and contents of the hen's egg would contain enough citric acid to provide all that contained in the embryo, for originally the shell of a 50 g. egg has 7·5 mg., the yolk 2·3 mg. and the white 0·3 mg. [Thunberg, 1941, 2]. In fact a newly hatched chick (Table 7) from an egg, which before incubation weighed 64·2 g., contained 4·56 mg. citric acid, or 3·55 mg. calculated for a 50 g. egg. Unincubated eggs of the same strain (R. I. R) contained, per 50 g. egg, 3·45 mg., and 21-day incubated, non-fertile, eggs 3·87 mg. citric acid (means of 3 each); while the shells of these eggs had only 2·18 and 1·95 mg., a much lower proportion than in Thunberg's experiments. The shell at hatching had 1·55 mg. in the example quoted in Table 7.

Equally important for the question of the origin of citric acid in the body, is a reconsideration of the work of Sherman *et al.* [1936] in the light of the present results. These authors conclude that 'consideration of the large amounts of citrate which are excreted by dogs on low citrate diet during prolonged alkalosis, and the absence of stores of preformed citrate in blood, liver, muscle and kidney, lead to the conclusion that the dog can synthesize citric acid'. Examination of the results of Sherman *et al.* [1936] shows that the three dogs studied by them excreted, over periods of from 18 to 45 days, 392, 335 and 608 mg. citric acid per kg. of dog. Such large amounts could not arise from preformed stores in tissues other than bone, but it seems possible that they might originate in the skeleton. Data for the dry weight of the total bones of the dog have not been found, but in a well fed rabbit the bones constitute 8·7 % of the body wt. [Aron, 1909, p. 196]. Assuming a similar value for the dog, this animal would have per kg. some 80–90 g. dry bone. If we take the value from Table 5 for the normal pup, as applying to the whole bone of the dog, and make an allowance for the fat content, these bones might have about 1 % citric acid. If these figures prove to be correct, the skeleton alone of dogs would contain about 800–900 mg. citric acid per kg. of dog, more than enough to cover the whole excretion of citrate observed by Sherman *et al.* [1936], provided that the loss of citric acid from the bones could reach 50 % of the total amount originally present. From this admittedly very rough calculation it appears doubtful if the evidence of these authors, unsupported by bone analyses, may be interpreted to prove the endogenous synthesis of citric acid in the dog. This important question could now be decided by further experiments in which the citrate of the bones is taken into account. The same authors' preliminary experiments on the effect of alkalosis on citrate excretion in the rat, however, suggest that this point could be more easily proved by the use of this species [cf. Sober *et al.* 1940]. This would be very desirable in order to establish that the synthesis of citrate which Krebs & Eggleston [1940] observed *in vitro* also occurs in the living animal.

Summary

1. The citric acid content of the solid tissues of several species of animals has been determined.

2. It has been found that the hard substance of bone contains a hitherto unsuspected store of citric acid, which may constitute some 70 % of that contained in the whole body. The marrow and cartilage contain much smaller amounts. Variations in the citrate content of bone, believed to be due to hormonal and dietary conditions, are reported. The variation of citrate greatly exceeds that of the other bone constituents, suggesting that the citrate is in a readily available form *in vivo*. *In vitro* it is not readily extracted from dried powdered bone by water, alcohol or ether.

3. Pure citric acid has been isolated from dried bone meal, and pure pentabromoacetone has been prepared from a trichloroacetic acid filtrate of bone meal in 80 % of the yield calculated from analysis. It is concluded that the substance estimated in bone is in fact citric acid.

4. The skin and hair have high contents of citric acid, which may arise, at least in part, from the sebaceous secretion: a high content was found in a human sebaceous cyst. The citrate present in hair is to a large extent extractable by water.

5. The very young (3- to 5-day) chick embryo is rich in citric acid, and the amount present is sufficient to combine with one-third of the total embryonic calcium. As development proceeds, Ca increases more rapidly than citrate.

6. Tumour tissue usually, though not invariably, contains much citric acid.

7. The need for revision of certain experiments on the citric acid balance in animals, caused by the present findings, is pointed out.

8. It is concluded that the existing hypotheses of 'citrate-like substances' being concerned in calcium metabolism receive their main experimental support from the present detection of citrate itself as a major constituent of bone.

I am greatly indebted to Mrs C. M. Burns for bone specimens and analyses; to Mr T. W. Smith, of Messrs Williamson and Corder, Newcastle, for information about bone meal and for a gift of this material; to the Pathology Department, University of Durham, for the use of their photometer; and to the Physicians and Surgeons of this Hospital for clinical material.

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Growth and Cell Replacement in Vitamin Research

XVI. Rachitis, prerachitis, and their causes

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Text includes 19 figures

(Received on January 7, 1942).

In the 8th and 9th reports of this series, experiments were described (1933) in which I attempted an analysis of the causation of rachitis on the basis of disturbed growth processes. It was shown at that time that "rachitis" is more than a consequence of vitamin D deficiency; it must have a much more complicated cause. To explain the histological changes, especially the classic work of Erdheim on "Rachitis and parathyroids" had to be taken into consideration. In it (p. 464) it had been shown that rachitis presuppose "growth", but that this growth derives from two mutually opposite processes - a decreased production compared to the norm, and a vascular and cellular resorption still more decreased compared to the production. The "elongation of the cartilaginous columnar layer" characteristic of the rachitic state, which can be seen in the x-ray picture also for instance as a cause of epiphyseal changes, proves to be primarily the result of the greatly decreased resportion. Although there is little replenishment, it outweighs the resorption, resulting in slow, imperfect growth. The rachitic bone is plump and thick.

In the former work I was able to show also that this decreased resorption was closely associated with a "metabolic weakness" or a "decreased tissue respiration", as was observed occasionally also in the history of the vertebrates in the case of saurians, for example, which changed from terrestrial to aquatic life and thus underwent a reduction in tissue respiration, besides finding a less varied mineral diet. This resulted in a fine-meshed bone structure, which could be produced experimentally by giving a diet high in phosphorus but low in calcium. As in the experiments of Stoelzner and Salge, where dogs were fed meat-fat resulting in "pseudorachitis", this led to exactly the same bone structures in rats. Since in these rats the bone marrow showed a fibroid structure, I called this "pseudorachitic" syndrome "fibroid malacia." True rachitis, on the other hand, comes about on the same basis of diminished respiration but reaches a different stage of bone formation - "osteoid". This evolutionally earlier formation presupposes the absence or restriction of phosphorus in the presence of much calcium. Absent is the formation of the bone cementing substance, without which no "bone capable of calcification" can form.

In man, such a fibroid picture is practically never found in this pure form. but only the "osteoid" rachitic picture. The reason for this is not that man could not react in such a way (*), but rather that his habits of living and eating tend by themselves to produce the causes of osteoid formation much more easily than the fibroid. It would be quite possible to bring about the abnormal picture artificially.

These two abnormal bone formations were thus regarded as a reversion to earlier evolutional stages, a view which in the area of the deficiency diseases has not yet asserted itself as an explanation of the morbid. But now that another ten years have gone by and many new findings have been added, it seemed to me that it was time to check these older observations with new methods.

A first reason that prompted me to do this was that rachitis could no be explained as lack of vitamin D alone. This has been set forth at length in a general study concerning this complex of problems (see Klin. Wschr., in course of publication).

Then also I was able to show in my own work that it is perfectly possible

for life to exist even if the metabolic processes occur at a minimum level (Report XIV). This lowest level of subsistence, termed mesotrophy, occurs when vitamin B_1 and potassium are present. All other vitamins and minerals may be absent. They become operative only when the substances formerly called HG factors and now "more thermostable growth substances" or procreative substances (**) are present in the diet.

Now since growth can occur when these growth substances are present in the diet, a further close relationship with the causation of rachitis existed. I had been able to show in many instances that vitamin D for example remains ineffective without growth substances.

Accordingly, the known rachitis diets had to be tested for the presence of growth substance. It was to be considered whether the growth substances may be stored from birth, as described in the 7th Report, also in the case of rachitis and act, so to speak, as "Deus ex machina". These questions had to be considered in connection with the mineral correlations. In short, numerous aspects could be dealt with so as to approach the basic problem of rachitis on a broader basis than the isolated idea that absence of an "antirachitic factor" leads to rachitis. This is in contradiction to the wealth of otherwise known concomitant facts.

In an introductory paper published in Klinische Wochenschrift, I showed that all the facts that have become known permit the idea that before the actual coming into being of the osteoid rachitic seams, a non-specific state first of all favors the genesis of rachitis. The term "prerachitis" was coined for this state still to be investigated. In this connection it was shown that young rats have a disposition for rachitis peculiar to them which is not present in older rats. For this, comparative tests between older and younger rats with the so-called rachitis diets had to be made.

We thus had many lines of approach in the exploration of the overall pic-

ture "rachitis". This paper reports on the first part of these experiments. The objective we have in mind is to find a real synthetic diet so as to eliminate the uncertainty factors of the present types of diet, as the rachitis diet at present most widely used can only standardize the vitamin D but does not serve to explain the overall picture involved in rachitis.

Method

1. For the study of the above discussed questions we started by comparing several known rachitis diets with one another, later modifying them.

a) McCollum 3143 (after Bomskov: Meth. d. Vitaminforsch, 1935, p. 89):

Wheat grits	33 parts
Corn grits	33 "
Wheat gluten	15 "
Gelatin	15 "
Common salt	1 "
$CaCO_3$	3 "

The wheat gluten is extracted with 96% alcohol for 12 hours. Per animal and per day first 5 g, later up to 15 g of the finished diet are given, so that there always remains an excess.

In Experiment 72, the following changes were made:

Instead of wheat grits and corn grits (Maizena), wheat grains and corn kernels; instead of wheat gluten, purified casein. Gelatin was increased to 18 parts.

b) Sherman-Pappenheim 84:

Wheat flour	95 parts
NaCl	2 "
Ca-lact.	2.9 "
Fe-citr.	0.1 "

2. The diets were given to young and older rats.

3. The original McCollum diet was supplemented with a calcium mixture of 28% Ca-citr. and 2% Ca-glycerinophosphorus. The same plus Vigantol, further

a combination product, Calcipot D, chosen because of its yeast content. Accordingly there were to be expected (1) growth substance effects and (2) concomitant effects of the B complex. (According to the manufacturer, Calcipot D consists of the above stated calcium mixture, addition of irradiated yeast containing 3600 biol. vitamin D units in 1.8 g, further as taste correctives sugar and cacao. According to de Rudder et al. this combination product has proved to be a good prophylactic. We therefore believed it to be very suitable for comparison with isolated vitamin D in Vigantol.

4. Test method.

In view of the entirely different objective, also a different test method than the usual was adopted. We chose the histological examination of the cartilaginousosseous interface of the ribs, as I had seen from earlier experiments (Reports VIII and IX) that they showed the finest reactions. In addition, the knee joints were x-rayed for comparison.

What made things difficult was that I could not breed my own rats in the rooms at my disposal. I had to use commercial rats. This uncertainty had to be accepted. But the animals came from a standard breed.

In order now to study the different stages of development of the anticipated changes, we treated the rats, whose weight was as nearly equal as possible, in each test series as follows:

Each series contained 7 rats. One of these was killed every fourth day, that is, on the 4th, 8th, 12th, 16th, 20th, 24th and 28th day. The knee joints of the dead animals were cut out and x-rayed in series, while the ribs were prepared for histological examination. According to the technique practiced in all test series of this project, the ribs 6 to 10 were cut out of the thorax by making an incision on the breastbone, then a cross cut of about 1½ cm over the cartilaginousosseous interface, an approximately square piece being obtained (***).

The ribs and knees were placed in 5% formalin. Before embedment they were put in Orth's mixture for 4 days. Then followed decalcification by suspension

in 5% nitric acid. The ribs stay 45 minutes, the knees 5 hours in the decalcifying solution; then they are put in sodium sulfate for 24 hours, then in running water for 24 hours. Afterwards 24 hours in 12.5% gelatin and 24 hours in 25% gelatin. Keeping in warm water (35-40°C), rinsing, and keeping in 5% formalin. Knees are put in the refrigerator in gelatin for 30 minutes, then also placed in 5% formalin. Brief watering before the frozen sections are prepared. Staining with hematoxylin-eosin. Thickness of the sections 15-20 micron.

Experiment 70

The first test series with the McCollum diet was divided into the following sub-groups:

1. Original (see above)
2. Addition of 3.6 mg calcium salt mixture daily.
3. Addition of 3.6 mg calcium salt mixture and Vigantol (commercial product, about 1 U.).
4. Addition of Calcipot D 12 mg daily.
5. Addition of 1/4 g wheat germ.
6. Addition of 1/4 g wheat germ and Calcipot D 12 mg daily.

All animals were weighed every fourth day. In part they lost weight at first, and then usually theygained. Table 1 shows the weight increases over the low point.

Table 1. Weight curves.

Group	4th	8th	12th	16th	20th	24th	28th day
McCollum original	R 2853	2854	2855	2856	2857	2858	2859
	-20	+ 4	+ 4	+ 10	+ 11	+ 6	+ 16
Calcium salt mix	R 2932	2933	2934	2935	2936	2937	2938
	+ 3	+ 10	+ 5	0	0	+ 5	+ 10
Calcium salt mix	R 2925	2926	2927	2928	2929	2930	2931
	+ 3	+ 4	+ 8	0	+ 10	+ 9	+ 8

Table 1 continued.

Calcipot D	R 2846	2847	2848	2849	2850	2851	2852
	0	- 6	+ 6	+ 7	+ 6	+ 8	+ 18
¼ g wheat germ	R 2881	2882	2883	2884	2885	2886	2887
	- 10	+ 6	+ 10	+ 6	+ 5	+ 12	+ 14
¼ g wheat germ	R 2874	2879	2875	2876	2877	2878	2880
	- 10	- 10	+ 14	+ 12	+ 11	+ 10	+ 25

It is found that the addition of the calcium salt mixture results in the lowest weight increase, which is even further reduced by addition of vitamin D (28th day). The increase is best when Calcipot D with ¼ g wheat germ is added.

The x-ray pictures (fig. 1) show - the photographs of the 16th to 28th day are reproduced - that a rachitis type enlargement of the epiphyseal line is found in the following four groups:

McCollum diet, from the 20th day;

McCollum diet and calcium mixture, from the 4th day;

McCollum, calcium mixture and Vitangol, from the 24th day;

McCollum and ¼ g wheat germ, from the 24th day.

No rachitis is found in the two Calcipot series.

Table 2. Response of the x-ray pictures.

	4th	8th	12th	16th	20th	24th	28th day
First series	(-)	(-)	(-)	(-)	+	+	+
2nd "	(+)	+	+	(+)	(+)	+	+
3rd "	(-?)	(-?)	-	(-)	-?	+	+
4th "	(-)	(-)	-	-	-	-	-
5th "	(-)	-?	-	-	-	+	+
6th "	(-)	(-)	-	-	-	-	-

+ = Rachitic changes.

In Table 2, the weight increases which are too small to be rated are bracketed, those recognizable as positive being marked by +.

It will be interesting to compare the x-ray findings on the knee joints with the histological findings on the ribs. For reasons of economy it is not possible to publish all the photographs. Instead, Table 3 lists the values found in the breeding capsule zone and in the columnar zone.

For comparison I refer to Table 2 in the 8th Report (vol. 170, p. 664).

The bracketed values denote the rats where the weight increase was too small. It is seen that the proliferation zone (breeding capsule zone) does not show values in any relation with the advancing rachitis, but that the depth of the columnar zone gives very unambiguous values. This is in agreement with the results reported by Erdheim. We may say:

The depth of the columnar layer is an exact expression of the severity of the rachitic changes.

We see that rather interesting values of the columna zone occur in the various groups:

1. Original diet; From the 20th day on, the zone increases progressively to 750 micron (Plate 1, fig. 2-13, 8th, 20th and 24th day).

2. With calcium mixture addition the highest value occurs in 8 days, so that all rats show rachitic values, despite insufficient weight increase.

3. Calcium mixture with Vigantol has the effect that to the 16th day perfectly normal conditions prevail, but then - delayed - an increase in length of the columnar zone occurs: Despite Vigantol, typical rachitic cartilage develops. The finding will be discussed below.

4. Calcipot D shows exactly the reverse: From initially high, suspicious values the columnar zone decreases to 85, that is, thenormal average value.

5. Wheat germ (¼ g) contains no antirachitic substances, that is, the growth substances contained therein cannot be regarded as effective by themselves (fig. 14).

Table 3. Measurement of the growth zones in the rib carilages. (values in μ)

	Prolif. zone	Columnar zone	Trabeculae		Prolif. zone	Columnar zone	Trabeculae
1. McCollumn (3143 (Original)				4. Addition of Calcipot D			
(2853)	(250)	(100)	(250)	(2846)	(315)	(250)	(150)
(2854)	(250)	(130)	(300)	(2847)	(335)	(200)	(215)
(2855)	(250)	(165)	(250)	2848	250	100	250
(2856)	(215)	(130)	(200)	2849	250	135	200
2857	250	585	350-700	2850	235	100	115
2858	250	500	100-150	2851	200	85	115
2859	250	750	500	2852	165	85	115
2. McCollumn 3143 + 3.6 mg Ca mix				5. Addition of wheat germ			
(2932)	(415)	(165)	(380)	(2881)	(250)	(85)	(250)
2933	300	850	250	2882	165	250	165
2934	415	200	250	2883	335	250	165
(2935)	(300)	(350)	(100-150)	2884	250	250	250
(2936)	(350)	(250)	(400)	2885	165	250	165
2937	200	600	250	2886	250	585	335
2938	250	405	165	2887*	250	585	335
3. McCollum 3143 + 3.5 mg Ca mix				6. Calcipot D + wheat germ			
(2925)	(300)	(200)	(165)	(2374)	(165)	(65)	(165)
(2926)	(250)	(200)	(250)	(2879)	(165)	(65)	(200)
2927	500	165	250	2875	335	150	150
(2928)	(165)	(165)	(335)	2876	285	135	165
2929	165	250	165	2877	165	85	165
2930	335	335	165	2878	165	100	250
2931	250	250	250	2880*	165	100	115

Table 3 continued.

Standard							
Diet X + ½ g yeast				Diet X + 1 g yeast			
2888	415	65	250	2890	415	100	250
2889	250	85	250	2891	330	100	330

* Fig. 14 & 15

First Series: McCollum diet, original.

Rat 2854 (fig. 2): Normal behavior of the cartilage columns on the 8th day.

Rat 2857 (fig. 3): On the 20th day, marked enlargementof the columnar zone with extensive distortions, irregular vessel cavities.

Rat 2858 (fig. 4): After 24 days marked "rachitis": Enlargement of the columnar zone, bloating. On the bone side, an irregular neoformation with osteoid seams.

Second Series: McCollum diet plus 3.6 mg calcium mixture daily.

Rat 2933 (fig. 5): Already on the 8th day, typical "rachitis", i.e. enlargement of the cartilage column zone and irregular formation at the marrow.

Rat 2936 (fig. 6) (20th day) and Rat 2938 (fig. 7) (28th day): Typical rachitis.

Third Series: McCollum diet, 3.6 mg calcium mixture and Vigantol (1.08 U.).

Rat 2926 (fig. 8): Still normal behavior on the 8th day.

Rat 2930 (fig. 9): On the 24th day an enlargement of the columnar zone begins, which with the secondary changes must be regarded as a mild rachitis.

Rat 2931 (fig. 10): On the 28th day: Rachitis well developed; the dose of Vigantol (1 U.) has failed.

Fourth Series: McCollum diet and Calcipot D.

Rat 2847 (fig. 11) (8th day), rat 2850 (fig. 12) (20th day) and rat 2852 (fig. 13) (28th day): all normal. On the 8th day possibly slight damage which is soon overcome.

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6. Wheat germ with Calcipot D results from the start in low values of the columnar zone remaining normal. Already after 4 days we have the low base value (fig. 15).

As to the trabecular zone, on the average it shows the lowest values with the protective supplements. Again an imperfect resorption would seem to be the cause of these elongations. The cause of the elongation of the trabecular zone has been clarified by Erdheim's analysis in his publication "Rachitis and parathyroid" (see above) (Euler's tooth research, referred to before, permits a still finer analysis of the modifications.)

The elongated cartilaginous columnar zone, proving rathitic bone, thus turns out to be the result especially of greatly reduced resorption rather than of increased neoformation. It follows that resorption must be the determining factor in the genesis of the rachitic bone. If it is weaker than the neoformation, we have inhibited epiphysis; if they are in equilibrium, normal growth results; if it becomes stronger, we have atrophic processes - the columnar zone shortens, is resorbed, and we get to the puely atrophic pictures as illustrated in earlier publications (vol. 153, p. 371; vol. 189, p. 195; vol. 167, p. 535).

Contrary to the earlier view that the so-called osteoid seams are calcium-depleted old bone - a view which has led to the prescription of calcium preparations in rickets - it has been proven by the analyses of M. B. Schmidt, Erdheim and others that osteoid seams are newly added bone matrix which, therefore, owes its formation to a productive activity of the osteoblasts. And since the bone itself is subject to intensive internal conversion, so that the addition takes place preferably from theoutside and through osteoblasts, while the removal is conducted from the inside through osteoclasts, there results also in the osseous part of the skeleton an antagonism between addition and consumption. The same applies concerning the ratio between these two properties as concerning the formation and resorption of cartilage (see above). We must assume, therefore, that in rachitis the osteoclast activity is reduced

more considerably than the osteo-blast acitivity. This centers into consideration for the causation of osteomalacia in adults.

Realizing, then, that growth substances are absolutely necessary for the formation of cartilage as well as of bone, we must come to the conclusion that they are involved in the causation of morbid tissue, whether they originate from the diets or have been stored in the body from before the test period or whether the young organism is able to synthesize them while the other is not. We shall see below that the latter assumption is the more probable. Proof, of course, can be furnished only when chemical substances are given in exactly measured quantity in a really correct synthetic diet and the excretion can be determined.

From the above test series it can be seen further that the addition of calcium in experimental rachitis of rats actually brings about a deterioration of the state and may lead so far that even the normally effective curative dose of vitamin D has only a postponing but not a definitely prophylactic significance.

Under this study program, a number of additional experiments have been carried out.

Experiment 71

In the same arrangement as Experiment 70, the Sherman-Pappenheim Diet 84 was tested. The result shows in the weight curves of the young animals (Table 4) that generally their gain was even somewhat better than with the McCollum diet, and that the unfavorable ratio phosphorus:calcium is harmful.

Were we to think that this definitely good weight increase should have been followed by a rachitis, this experiment would be a complete disappointment, for it can be seen from Table 5 that the cartilaginous columnar zone remained low everywhere and no rachitis developed. (* Here again Euler's research on teeth is informative.)

With respect to growth, therefore, the two diets are completely different. It is not yet clear whether a modification of the process of production or of resorption has occurred here. This would require further tets. He cannot be sure that the Sherman-Pappenheim Diet 84 is rachitogenic. Unknown additional factors must be investigated.

Table 4. Weight increase.

Group	4th	8th	12th	16th	20th	24th	28th day
Sherman-Pappenheim 84,	R 2839	2840	2841	2842	2843	2844	2845
	- 10	+ 3	+ 8	+ 10	+ 10	+ 8	+ 6
Calcium salt mixture	R 2924	2918	2919	2920	2921	2922	2923
	- 10	+ 3	+ 5	+ 10	+ 15	+ 19	+ 23
Calcium salt mixture and Vigantol	R 2911	2912	2913	2914	2915	2916	2917
	+ 1	+ 7	+ 15	+ 7	+ 17	+ 20	+ 25
Calcipot D	R 2832	2833	2834	2835	2836	2837	2838
	- 5	0	+ 5	+ 11	- 10	+ 5	+ 10
½ g wheat germ	R 2867	2868	2869	2870	2871	2872	2873
	- 20	+ 8	+ 5	+ 8	+ 12	+ 15	+ 10
½ g wheat germ Calcipot D	R 2860	2861	2862	2863	2864	2865	2867
	- 15	+ 3	+ 7	+ 13	+ 7	+ 22	+ 20

Table 5. Measurement of the growth zones in the rib carbilages.(Values in μ)

	Prolif. zone	Columnar zone	Trabeculae		Prolif. zone	Columnar zone	Traveculae
Serman-Pappenheim 84				+ Calcipot D			
2839	165	100	335	2832	250	65	200
2840	300	65	200	2833	165	65	165
2841	200	100	250	2834	250	65	200
2842	335	100	250	2835	250	115	250
2843	200	35	85	2836	165	0	0
2844	250	100	115	2837	330	100	165
2845	200	50	100	2838	165	0	0

Table 5 continued.

+ 3.6 mg calcium mix				+ wheat germ			
2924	250	100	335	2867	250	85	165
2918	250	85	165	2868	250	85	200
2919	330	100	165	2869	250	65	165
2920	250	150	330	2870	250	115	165
2921	165	200	330	2871	200	65	165
2922	250	200	250	2872	250	100	165
2923	250	100	250	2872	165	130	165
+ Ca mix + Vigantol				+ Calcipote D + wheat germ			
2911	250	100	250	2860	300	50	250
2912	330	65	330	2861	250	65	200
2913	200	100	250	2862	250	50	165
2914	250	150	200	2863	250	100	250
2915	300	50	200	2864	165	130	165
2916	200	100	200	2865	165	100	165
2917	250	115	165	2866	200	50	130
Standard							
Diet X + ½ g yeast				Diet X + 1 g yeast			
2888	415	65	250	2890	415	100	250
2889	250	85	250	2891	330	100	330

Experiment 72a

Experiments with older rats (initial weight 70 - 105 g)

The Sherman-Pappenheim Diet 84 was used in the same manner as in Exp. 71.

With McCollum diet we find at first, up to 3 weeks, a fairly great weight increase, then rapid decline and death in 8-10 weeks.

Experiment 72b

In these tests, 12 mg of Calcipot D were added to the two diet forms daily. It appears from Curve 2 (McCollum diet) and Curve 3 (Sherman-Pappenheim diet) that this changes nothing in the shape of the curves leading to loss of weight and death.

(Broken line: Ideal growth curve)

Histologically all rats of these test series show pure atrophies. There are no signs of a rachitis having been overcome during the experiment. (* Euler's research on teeth is again informative.)

Possible causes are: Lack of growth substances, amino acids, minerals.

Perhaps wheat gluten contains such substances, e.g. an as yet unknown factor which stimulates the formation of bone matrix? This must be decided in further experiments.

By comparison, the normal weight curve under a complete diet is shown along with the weight increase under McCollum Diet 3143 in Curve 4.

Evaluation of Experiment 72a and b

We may, at first, be inclined to attribute the different shape of the weight curves under the action of the rachitis diets in these two test divisions to the modification of the McCollum diet. This is not very likely, however, because the Sherman-Pappenheim diet shows the same weight loss although it was not modified. It would seem more probable, therefore, that the greater age of the animals and therefore the higher requirements of the more mature animal are the endogenic cause of the different reaction. Since in addition the grain supplement must undoubtedly be much more complete than the use of grit, one should rather expect an improvement of the effect. The transformation of wheat gluten into casein could at any rate be a possible cause. The research is being continued.

The greater likelihood is that due to their higher requirements the older rats evidence the deficiencies of the rachitis forms more clearly than was possible in the younger rats. In the younger rats, the cooperation of reserve growth substances from previous feeding or their production in small quantity is the most probable.

A supplement of 12 mg of Calcipot D causes no improvement. Whether higher supplements achieve an improvement we have not yet been able to investigate. On the basis of earlier experience, however, it may be expected that the yeast component would have to be increased considerably to produce an effect on the older individual. Growth substances evidently do nothave that trace effect that vitamin D has.

Discussion of the results

1. Both tested rachitis diets indicate that they are highly imcomplete also in other respects, especially the Sherman-Pappenheim diet; compared with the standard, the rats lag far behind in weight. Older rats lose weight drastically, develop an atrophy of high degree and die.

It follows that the rachitis diets must have not only a vitamin D deficiency but also other deficiencies. These have been pointed out often before. I refer to Bomskov's compilation. The environmental effects resulting from these deficiencies are one part - that due to nutrition - of the prerachitic state.

2. Older rats develop an atrophy under a slightly modified McCollum diet and under unmodified Sherman-Pappenheim diet where young rats still get rachitis. From the difference between these two reactions of the ages it may be possible to explore the second - endogenic - component of the prerachitic state.

3. Pure vitamin D has a postponing prophylactic effect only if too much calcium mixture is given. It will be necessary to investigate the evolutional deeper causes of rachitis disposition. It may be necessary to make comparative studies on descending levels of the animal kingdom down to the cartilaginous fishes in order to explore chemically the relation phosphorus to calcium and the relationship with vitamin D. It appears certain that these processes are closely linked with the development of the hormone glands.

4. The histological examination of the growth zones enables us to study the finer processes. As in the 7th Report (p. 228), the disturbances of the vascular resorption in these preparations were striking - anemia of the capillaries, often almost complete collapse. On the basis of earlier findings it may be assumed that here an additional vitamin A deficiency is involved. And it is not unlikely that the rachitic's increased disposition for infection has something to do with this disturbed capillary function and the consequences thereof.

5. Also it should be investigated whether a possibility of life similar to mesotrophy at the lowest limit could be sufficient to explain the prerachitic

state.

6. In practice it can be concluded from these experiments already that the isolated application of vitamin D can be sufficiently prophylactic. But this prophylaxis cannot eliminate the damge done by the other deficiencies of the diet demonstrated above, which lead to prerachitis. For this, combined preparations are definitely more promising.

7. Experimental rachitis research thus far, therefore, can only standardize the vitamin D. The whole scope of "rachitis" and the explanation of the diseases resembling it must be attempted by a major expansion of the research methods. Nor are the older test proecures fine enough to show the finer partial phases of the growth process.

The rachitis experiments must always be perfected by taking into consideration the tooth and jaw modifications. Express reference is made to Euler's research (see 18th report).

We must cut loose from the previous one-track vitamin D research and turn to a more comprehensive rachitis research as such. The 8th and 9th reports showed the way. In the next report, the formation of antibodies under mesotrophic diet will be described.

(Completed on January 5, 1942)

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(*) We have a similar formation in "osteodystophia fibrosa".

(**) It might be well to call the animal procreative substances "auxons" while continuing to use for the plant substances the term "auxins" introduced by Kögl. This would emphasize the relationship and difference.

(***) Jaws and teeth were sent to Prof. Euler, Institute of Dentistry, University of Breslau. These findings will be published separately.

Translated by Carl Demrick Associates, Inc./LH/db

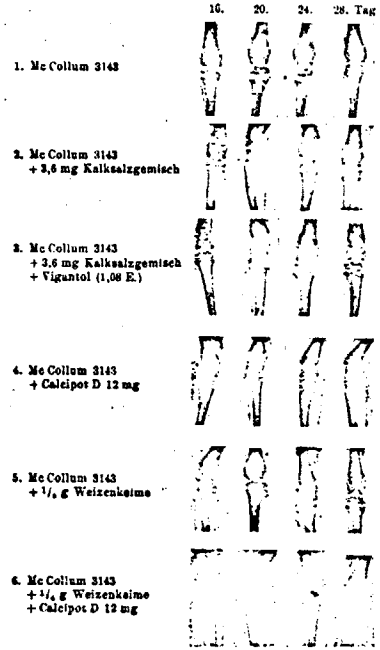


Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

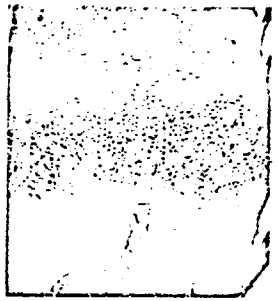


Fig. 6

Fig. 7

Fig. 8

Fig. 9

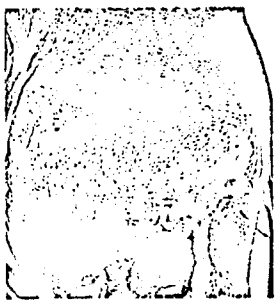


Fig 10

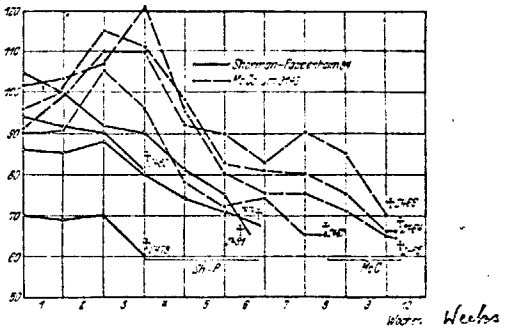
Fig. 11

Fig. 12

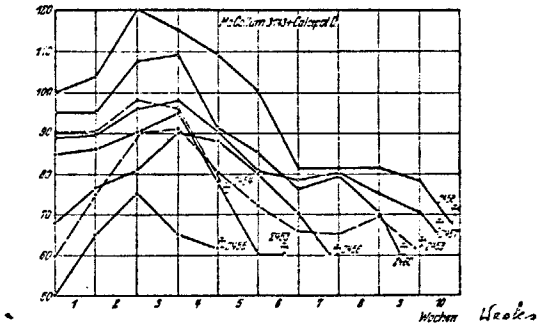
Fig. 13

Fig. 14

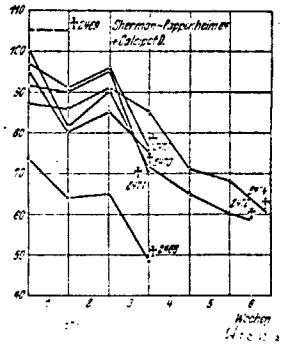
Fig. 15



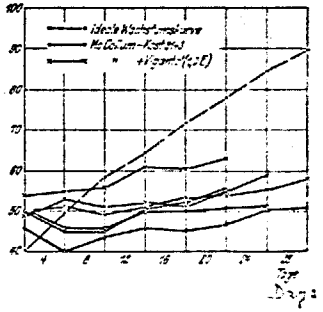
Curve 1



Curve 2



Curve 3



Curve 4

bei Rückflußkühlung entsteht der β-„Glphch.". Der so gewonnene Cholinester der β-Glycerinphosphorsäure schmiltz bei 104—105° C, enthält weder freies Cholin, noch freie „Glph.", welche nach Alkalisierung mit Ammoniumhydroxyd nachweisbar wäre.

3. Die Wirkung des Esters wurde am Verhalten des Blutdruckes von Katzen, an den Darmbewegungen von Katzen (beide in Chloralosenarkose) und in Froschherzversuchen untersucht.

Die Wirkung der zweierlei hergestellten Ester war annähernd identisch und entsprach ungefähr der Wirkung des freien Cholins, obwohl die vorher vorgenommene Florence-Probe mit beiden Estern negativ ausfiel. Während große Dosen von Cholinchlorid (10 mg) ohne eine Vorbehandlung mit Atropin die sogenannte Nikotinwirkung aufwiesen, ließ sich diese Erscheinung unter der Wirkung keines der Ester beobachten. Ihre Wirkung blieb auch nach Nebennierenentfernung unverändert. Nach Atropinbehandlung rief auch der synthetische Ester eine milde umgekehrte Reaktion vor. Nach Dekapitation wurde der Blutdruck von keinem der Ester gesenkt. Die Darmbewegungen wurden durch beide Esterarten lebhafter, der Darmtonus nahm zu.

Verfasser nehmen an, daß der Ester nach seinem Eintritt in den Organismus durch Cholinphosphatase zerlegt wird. Auf diese Weise entsteht freies Cholin, dessen pharmakologische Wirkung zur Geltung kommt.

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(Direktor: Prof. Dr. Werner Kollath.)

Wachstum und Zellersatz in der Vitaminforschung.

XVI. Mitteilung: Rachitis, Prärachitis und ihre Ursachen.

Von

Werner Kollath,

unter Mitarbeit von

Johannes Garbe, Eva-Dora Schröder, Gustav Schunk und Emmy Thierfelder.

Mit 19 Textabbildungen.

(Eingegangen am 7. Januar 1942.)

In der VIII. und IX. Mitteilung dieser Arbeitenreihe wurden (1933) Versuche beschrieben, in denen ich eine Analyse der Rachitisentstehung auf Grund der gestörten Wachstumsprozesse versuchte. Es konnte damals gezeigt werden, daß die „Rachitis" nicht nur als Folge des Vitamin D-Mangels zu betrachten war, sondern eine wesentlich kompliziertere Ursache haben müsse. Vor allem mußte zur Erklärung der histologischen Veränderungen die klassische Arbeit von Erdheim über „Rachitis und Epithelkörperchen" berücksichtigt werden. In dieser war (S. 464) gezeigt, daß Rachitis „Wachstum" voraussetzt, daß dieses Wachstum aber aus zwei einander entgegengesetzten Prozessen entsteht: einer in Vergleich zur Norm gesunkenen Produktion, und einer in Vergleich zur Produktion noch stärker gesunkenen vaskulären und zellulären Resorption. Die für den rachitischen Zustand kennzeichnende „Verlängerung der Knorpelsäulenschicht", wie sie z. B. auch als Ursache der Epiphysenveränderungen im Röntgenbild erkennbar ist, erweist sich in erster Linie als Folge der stark gesunkenen Resorption. Obwohl der Nachschub schwach ist, überwiegt er doch die Resorption und es kommt zu einem schleppenden, unvollkommenen Wachstum. Der rachitische Knochen wird plump und dick.

Ich konnte in jenen Arbeiten auch zeigen, daß diese gesunkene Resorption eine enge Parallele mit einer „Stoffwechselschwäche" oder einer „gesunkenen Gewebsatmung" aufwies, wie sie z. B. auch in der Geschichte der Wirbeltiere gelegentlich bei Sauriern beobachtet wurde, die vom Land- zum Wasserleben übergingen und nun einerseits eine verminderte Gewebsatmung erfuhren, andererseits eine einseitigere Mineralkost zu sich nehmen konnten. Dabei entstand ein feinmaschiges Knochenwerk, das sich experimentell dadurch erzeugen ließ, daß phosphorreiche, aber kalkarme Kost gegeben wurde. Diese führte, wie in den Versuchen von Stoelzner und Salge, in denen Hunde mit Fleisch-Fett gefüttert wurden, wobei „Pseudorachitis" entstand, zu genau den gleichen Knochen-

Col. Otrak 489

strukturen bei Ratten. Da das Knochenmark bei diesen Ratten eine fibroide Struktur aufwies, nannte ich dies „pseudorachitische" Bild „fibroide Malazie". Die echte Rachitis hingegen entsteht auf der gleichen Grundlage des abgeschwächten Atmungsvorganges, erfährt aber eine andere Stufe der Knochenbildung: es entsteht „Osteoid". Und diese entwicklungsgeschichtlich frühere Bildung hat zur Voraussetzung die Abwesenheit bzw. Einschränkung des Phosphors bei Kalkreichtum. Es fehlt die Bildung der Knochenkittsubstanz, ohne die kein „der Verkalkung fähiger Knochen" entstehen kann.

Nun findet man ein derartig fibroides Bild beim Menschen in dieser reinen Form praktisch niemals, sondern nur das „osteoide" rachitische Bild. Das liegt nicht daran, daß der Mensch etwa nicht derartig reagieren könnte *, sondern daran, daß seine Lebens- und Ernährungsgewohnheiten von selbst aus dazu neigen, daß die Ursachen der osteoiden Bildung weit leichter eintreten, als die der fibroiden. Künstlich könnte man das abnorme Bild durchaus hervorrufen.

Diese beiden abnormen Knochenbildungen wurden somit als Rückfall in entwicklungsgeschichtliche Vorstufen betrachtet, eine Erklärungsform des Krankhaften, die sich im Bereich der Mangelkrankheiten bisher noch nicht durchgesetzt hat. Nachdem aber nun weitere 10 Jahre ins Land gegangen sind und zahlreichere neuere Erkenntnisse hinzukamen, schien es mir an der Zeit, diese alten Beobachtungen mit neuen Mitteln nachzuprüfen.

Zunächst bestimmte mich dazu, daß es nicht gelungen ist, die Rachitis allein durch Fehlen von Vitamin D zu erklären. Das ist in einer allgemeinen Studie zu diesem Fragenkomplex eingehend dargelegt worden (s. Klin. Wschr., z. Z. im Druck).

Sodann aber konnte ich in meinen eigenen Arbeiten zeigen, daß es durchaus möglich ist, daß das Leben besteht, wenn auch die Stoffwechselprozesse an der unteren Grenze der Norm verlaufen (XIV. Mitteilung). Diese, als Mesotrophie bezeichnete unterste Lebensmöglichkeit tritt ein, wenn Vitamin B_1 und Kalium anwesend sind. Alle anderen Vitamine und Mineralien können dabei fehlen. Sie werden nur wirksam, wenn die früher als HG-Faktoren, jetzt als „hitzestabilere Wuchsstoffe" bzw. Vermehrungsstoffe ** bezeichneten Substanzen in der Kost vorhanden sind.

Da nun Wachstum eintreten kann, wenn diese Wuchsstoffe in der Kost vorhanden sind, bestand eine weitere enge Beziehung zur Rachitisentstehung. Ich hatte vielfach zeigen können, daß z. B. ohne Wuchsstoffe Vitamin D unwirksam bleibt.

Man mußte nun die bekannten Rachitisdiäten auf Wuchsstoffanwesenheit prüfen. Man mußte erwägen, ob die Wuchsstoffe in ähnlicher Weise,

* In der „Osteodystrophia fibrosa" haben wir eine ähnliche Bildung.

** Es wäre vielleicht zweckmäßig, die tierischen Vermehrungsstoffe als „Auxone" zu bezeichnen, während die pflanzlichen die von Kögl eingeführte Bezeichnung „Auxine" beibehalten. Verwandtschaft und Verschiedenheit werden dadurch betont.

wie in der VII. Mitteilung beschrieben, auch bei Rachitis von der Mutter her gespeichert sein können und — gewissermaßen als deux ex machina — wirken. Man mußte diese Fragen in Beziehung zu den Mineralkorrelationen bringen. Kurz, es ergaben sich zahlreiche Aufgaben, aus denen das Gesamtproblem der Rachitis in umfassenderer Weise angegangen werden konnte, als mit der isolierten Vorstellung, Fehlen eines „antirachitischen Faktors" führe zu Rachitis. Das widerspricht der Fülle der sonst bekannten Begleitumstände.

In einer einführenden, in der Klin. Wschr. veröffentlichten Arbeit habe ich gezeigt, daß alle bekannt gewordenen Tatsachen den Gedanken erlauben, daß vor dem eigentlichen Werden der osteoiden rachitischen Säume ein unspezifischer Zustand das Werden der Rachitis überhaupt erst begünstigt. Für diesen näher zu erforschenden Zustand wurde die Bezeichnung „Prärachitis" geprägt. Es wurde dabei auch gezeigt, daß jugendliche Ratten eine ihnen eigene Disposition zur Rachitisentstehung haben, und daß diese bei älteren Ratten nicht mehr vorliegt. Vergleichende Untersuchungen zwischen älteren und jüngeren Ratten bei Verfütterung der sogenannten Rachitiskosten waren dazu notwendig.

Es gab also eine ganze Anzahl von Möglichkeiten, die neuen Gesichtspunkte zur Erforschung des Gesamtbildes „Rachitis" experimentell zu erproben. Über den ersten Teil dieser Versuche soll hier berichtet werden. Das vorschwebende Ziel heißt, eine wirkliche synthetische Kost zu finden, damit diejenigen Unsicherheitskomponenten, die den bisherigen Kostformen anhaften, beseitigt werden. Denn, die bisherige am meisten benutzte Rachitiskost ist nur geeignet, das Vitamin D zu standardisieren, aber nicht, das Gesamtbild Rachitis aufzuklären.

Methodik.

1. Zum Studium der oben besprochenen Fragen gingen wir davon aus, verschiedene bekannte Rachitisdiäten erstens untereinander zu vergleichen und sie zweitens zu modifizieren.

a) McCollum 3143 (nach Bomskov: Meth. d. Vitaminforsch. 1935, S. 89):

Weizengrieß	33	Teile
Maisgrieß	33	„
Weizenkleber	15	„
Gelatine	15	„
Kochsalz	1	„
$CaCO_3$	3	„

Der Weizenkleber wird 12 Stunden mit 96%igem Alkohol extrahiert. Pro Tier und Tag werden anfangs je 5 g, später bis zu 15 g der fertigen Diät gegeben, so daß stets ein Überschuß bleibt.

In dem Versuch 72 wurden folgende Veränderungen getroffen:

Statt Weizengrieß und Maisgrieß (Maizena), Weizenkörner und Maiskörner; statt Weizenkleber gereinigtes Casein. Gelatine wurde auf 18 Teile erhöht.

b) Sherman-Pappenheim 84:

Weizenmehl	95	Teile
NaCl	2	„
Ca-lact.	2,9	„
Fe-citr.	0,1	„

2. Die Diäten wurden bei jungen und älteren Ratten gegeben.

3. Zur Original-Mc Collum-Kost wurden Zulagen gegeben: ein Kalkgemisch aus 28% Ca-citr. und 2% Ca-glycerinophosphor. Dasselbe plus Vigantol, ferner ein Kombinationspräparat, Calcipot D, das wegen seines Hefegehalts gewählt wurde. Demnach ließ es 1. Wuchsstoffwirkungen erwarten, 2. Mitwirkungen des B-Komplexes. (Calcipot D besteht nach Angaben der Firma aus dem oben angegebenen Kalkgemisch, Zusatz von bestrahlter Hefe, die in 1,8 g 3600 biol. Vitamin D-Einheiten enthält, ferner als Geschmackskorrigentien Zucker und Kakao. Dies Kombinationspräparat soll sich nach de Rudder, Diener, Gierthmühlen, Techentin, Groetschel, Winkler und Dressen prophylaktisch gut bewährt haben. Es schien uns deshalb als Vergleichsobjekt zum isolierten Vitamin D im Vigantol sehr geeignet.

4. Testverfahren.

In Anbetracht des gänzlich anderen Zieles wurde auch ein anderes Testverfahren als das übliche benutzt. Gewählt wurde die histologische Untersuchung der Knorpelknochengrenze der Rippen, da ich aus den früheren Versuchen (VIII. und IX. Mitteilung) ersehen hatte, daß sie die feinsten Reaktionen erkennen ließen. Daneben wurden zum Vergleich die Kniegelenke geröntgt.

Eine Erschwerung trat dadurch ein, daß meine Arbeitsräume die Benutzung einer eigenen Rattenzucht nicht gestatteten. Infolgedessen wurde die Verwendung von Handelsratten erforderlich. Diese Unsicherheit mußte in Kauf genommen werden. Die Tiere entstammten aber einer einheitlichen Zucht.

Um nun die verschiedenen Entwicklungsstadien der zu erwartenden Veränderungen zu studieren, gingen wir so vor, daß die mit möglichst gleichem Gewicht eingesetzten Ratten in jeder Versuchsreihe in folgender Weise behandelt wurden:

In jede Reihe kamen 7 Ratten von möglichst gleichem Gewicht. Von diesen wurde jeden 4. Tag eine Ratte getötet, also am 4., 8., 12., 16., 20., 24. und 28. Tag. Die Untersuchung erfolgte nun so, daß von den toten Tieren die Kniegelenke herauspräpariert und reihenweise geröntgt wurden. Die Rippen aber wurden für die histologische Untersuchung vorbereitet. Entsprechend der in allen diesen Versuchsreihen dieser Arbeitenfolge geübten Technik wurden aus der Brustwand die Rippen 6—10 herausgeschnitten, so daß am Brustbein ein Einschnitt gemacht wurde, sodann ein etwa $1^1/_2$ cm-Querschnitt über die Knorpel-Knochengrenze geführt wurde und nun ein annähernd quadratförmiges Stück gewonnen wurde*.

Rippen und Knie wurden in 5%iges Formalin gelegt. Vor dem Einbetten kamen sie 4 Tage in Orthsches Gemisch. Hierauf erfolgte Entkalkung in 5%iger

** Kiefer und Zähne wurden an Herrn Prof. Euler, Zahnärztliches Institut der Universität Breslau, geschickt. Die Veröffentlichung dieser Befunde erfolgt gesondert.

Salpetersäure, durch Einhängen. Rippen verbleiben 45 Minuten, Knie 5 Stunden in der Entkalkungslösung; dann kommen sie 24 Stunden in Natriumsulfat, dann 24 Stunden in fließendes Wasser. Darauf kommen sie 24 Stunden in 12,5%ige Gelatine, dann 24 Stunden in 25%ige Gelatine. Aufbewahren in warmem Wasser (35—40° C), Abspülen und Aufbewahren in 5%igem Formalin. Knie werden in Gelatine 30 Minuten in den Eisschrank gestellt, dann auch in 5%iges Formalin gelegt. Vor dem Anfertigen der Gefrierschnitte wird kurz gewässert. Färbung Hämatoxylin-Eosin. Dicke der Schnitte 15—20 μ.

Versuch 70.

Die 1. Versuchsreihe mit der Mc Collum-Kost zerfiel in folgende Untergruppen:

1. Original (s. oben).
2. Zugabe von 3,6 mg Kalksalzgemisch täglich.
3. Zugabe von 3,6 mg Kalksalzgemisch und Vigantol (Handelsware. etwa 1 E).
4. Zugabe von Calcipot D 12 mg täglich.
5. Zugabe von $^1/_4$ g Weizenkeimen.
6. Zugabe von $^1/_4$ g Weizenkeimen und Calcipot D 12 mg täglich.

Alle Tiere wurden jeden 4. Tag gewogen. Teilweise erfuhren sie zuerst einen Gewichtsverlust, dann stiegen sie meist an. Die Gewichtszunahmen gegenüber dem Tiefpunkt sind in der Tabelle 1 zusammengefaßt.

Tabelle 1. Verhalten der Gewichtskurven.

Gruppe	4.	8.	12.	16.	20.	24.	28. Tag
Mc Collum Original	R 2853	2854	2855	2856	2857	2858	2859
	− 20	+ 4	+ 4	− 10	+ 11	+ 6	+ 16
Kalksalzgemisch	R 2932	2933	2934	2935	2936	2937	2938
	+ 3	+ 10	+ 5	0	0	+ 5	+ 10
Kalksalzgemisch und Vigantol	R 2925	2926	2927	2928	2929	2930	2931
	+ 3	+ 4	+ 8	0	+ 10	+ 9	+ 8
Calcipot D	R 2846	2847	2848	2849	2850	2851	2852
	0	− 6	+ 6	+ 7	+ 6	+ 8	+ 18
$^1/_4$ g Keime	R 2881	2882	2883	2884	2885	2886	2887
	− 10	+ 6	+ 10	+ 6	+ 5	+ 12	+ 14
$^1/_4$ g Keime Calcipot D	R 2874	2879	2875	2876	2877	2878	2880
	− 10	− 10	+ 14	+ 12	+ 11	+ 10	+ 25

Es ergibt sich, daß die Zugabe des Kalksalzgemisches die niedrigste Gewichtszunahme zur Folge hat, die durch Zugabe von Vitamin D sogar noch erniedrigt wird (28. Tag). Am besten ist die Zunahme, wenn Calcipot D mit $^1/_4$ g Keimen zugegeben wird.

Die Röntgenbilder (Abb. 1), es sind die Aufnahmen vom 16.—28. Tag abgebildet, lassen nun erkennen, daß eine als Rachitis anzusprechende Verbreiterung der Epiphysenlinie sich in folgenden 4 Gruppen findet:

Abb. 1.

Tabelle 2. Das Verhalten der Röntgenbilder.

	4.	8.	12.	16.	20.	24.	28. Tag
1. Reihe	(−)	(−)	(−)	(−)	+	+	+
2. „	(+)	+	+	(+)	(+)	+	+
3. „	(− ?)	(− ?)	−	(−)	?	+	+
4. „	(−)	(−)	−	−	−	−	−
5. „	(−)	− ?	−	−	−	+	+
6. „	(−)	(−)	−	−	−	−	−

+ = rachitische Veränderungen.

Mc Collum-Kost ab 20. Tag,
„ und Kalkgemisch ab 4. Tag,
„ „ „ und Vigantol ab 24. Tag,
„ „ 1/4 g Weizenkeime ab 24. Tag.

Bei den beiden Calcipotreihen findet sich keine Rachitis.

In der Tabelle 2 sind die infolge zu geringer Gewichtszunahme nicht zu bewertenden Ratten eingeklammert, die als positiv erkennbaren mit + angegeben.

Es ist nun von Interesse, mit den Röntgenbefunden bei den Kniegelenken die histologischen Befunde bei den Rippen zu vergleichen. Aus Sparsamkeitsgründen muß von der Veröffentlichung sämtlicher Aufnahmen abgesehen werden. Statt dessen sind in der Tabelle 3 die Meßwerte zusammengefaßt, die die Brutkapselzone sowie die Säulenzone ergaben.

Zum Vergleich verweise ich auf Tabelle 2 in der VIII. Mitteilung (Bd. 170, S. 664).

Die eingeklammerten Werte bezeichnen die Ratten mit zu geringen Gewichtszunahmen. Man erkennt, daß die Wucherzone (Brutkapselzone) keine Werte aufweist, die einen Zusammenhang mit der fortschreitenden Rachitis erkennen lassen, daß hingegen die Tiefe der Säulenzone

Tabelle 3. Messung der Wachstumszonen in den Rippenknorpeln. (Werte in μ.)

	Wucherzone	Säulenzone	Trabekel
1. Mc Collum 3143 (Original)			
(2853)	(250)	(100)	(250)
(2854)	(250)	(130)	(300)
(2855)	(250)	(165)	(250)
(2856)	(215)	(130)	(200)
2857	250	585	350—700
2858	250	500	100—150
2859	250	750	500
2. Mc Collum 3143 + 3,6 mg Kalkgemisch.			
(2932)	(415)	(165)	(380)
2933	300	850	250
2934	415	200	250
(2935)	(300)	(350)	(100—150)
(2936)	(350)	(250)	(400)
2937	200	600	250
2938	250	405	165
3. Mc Collum 3143 + 3,6 mg Kalkgemisch + Vigantol.			
(2925)	(300)	(200)	(165)
(2926)	(250)	(200)	(250)
2927	500	165	250
(2928)	(165)	(165)	(335)
2929	165	250	165
2930	335	335	165
2931	250	250	250
Norm!			
Diät X + 1/2 g Hefe.			
2888	415	65	250
2889	250	85	250
4. Zugabe von Calcipot D.			
(2846)	(315)	(250)	(150)
(2847)	(335)	(200)	(215)
2848	250	100	250
2849	250	135	200
2850	235	100	115
2851	200	85	115
2852	165	85	115
5. Zugabe von Weizenkeimen.			
(2881)	(250)	(85)	(250)
2882	165	250	165
2883	335	250	165
2884	250	250	250
2885	165	250	165
2886	250	585	335
2887 *	250	585	335
6. Calcipot D + Weizenkeime.			
(2874)	(165)	(65)	(165)
(2879)	(165)	(65)	(200)
2875	335	150	150
2876	285	135	165
2877	165	85	165
2878	165	100	250
2880 *	165	100	115
Diät X + 1 g Hefe.			
2890	415	100	250
2891	330	100	330

* Abb. 14 und 15.

ganz eindeutige Werte ergibt. Das stimmt genau mit den von Erdheim mitgeteilten Meßergebnissen überein. Man kann sagen:

Die Tiefe der Säulenschicht ist ein genauer Ausdruck der Schwere der rachitischen Veränderungen.

Man erkennt nun, daß in den einzelnen Gruppen recht interessante Werte der Säulenzone auftreten:

1. Originalkost: Fortschreitend nimmt vom 20. Tage ab die Zone bis 750 μ zu (Tafel 1, Abb. 2—13, 8., 20. und 24. Tag).

2. Bei Kalkgemisch-Zugabe findet sich der höchste Wert bereits bei 8 Tagen, so daß alle Ratten, trotz unzureichender Gewichtszunahme doch rachitische Werte zeigen.

3. Kalkgemisch mit Vigantol wirkt nun so, daß bis zum 16. Tage völlig normale Verhältnisse herrschen, dann aber tritt — verspätet —

Mc Collum 3143 Original.

Abb. 2. Ratte 2854. 8. Tag.

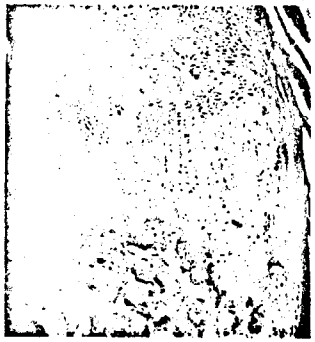

Abb. 3. Ratte 2857. 20. Tag.

Abb. 4. Ratte 2858. 24. Tag.

1. Reihe: Mc Collum-Kost, Original.

Ratte 2854 (Abb. 2): Normales Verhalten der Knorpelsäulen am 8. Tag.

Ratte 2857 (Abb. 3): Am 20. Tag findet sich eine ausgeprägte Vergrößerung der Säulenzone mit ausgedehnten Verwerfungen, unregelmäßigen Gefäßräumen.

Ratte 2858 (Abb. 4): Nach 24 Tagen ausgeprägte „Rachitis": Vergrößerung der Säulenzone, Auftreibung. Seitens des Knochens findet sich eine unregelmäßige Neubildung mit osteoiden Säumen.

2. Reihe: Mc Collum-Kost mit Zugabe von 3,6 mg Kalkgemisch täglich.

Ratte 2933 (Abb. 5): Bereits am 8. Tage findet sich typische „Rachitis", d. h. Vergrößerung der Knorpelsäulenzone und unregelmäßige Bildung am Mark.

Mc Collum 3143 + 3,6 mg Kalksalzgemisch.

Abb. 5. Ratte 2933. 8. Tag.

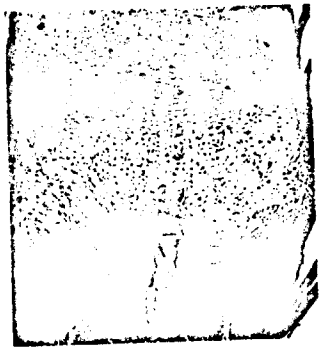

Abb. 6. Ratte 2936. 20. Tag.

Abb. 7. Ratte 2938. 28. Tag.

Ratte 2936 (Abb. 6) (20. Tag) und Ratte 2938 (Abb. 7) (28. Tag): typische Rachitis.

3. Reihe: Mc Collum-Kost, 3,6 mg Kalkgemisch und Vigantol (1,08 E.).

Ratte 2926 (Abb. 8): Es besteht am 8. Tag noch normales Verhalten.

Mc Collum 3143 + Kalksalzgemisch + 1 E Vigantol Handelsware.

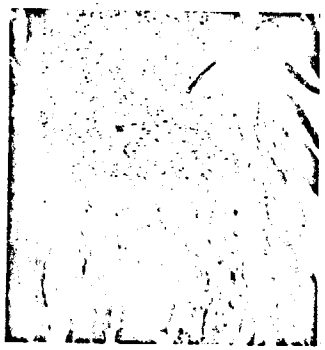

Abb. 8. Ratte 2926. 8. Tag.

Abb. 9. Ratte 2930. 24. Tag.

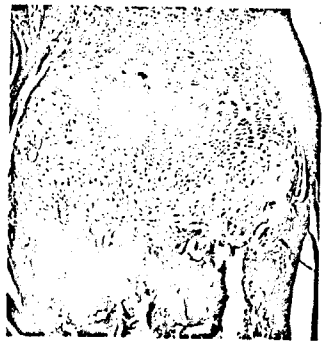

Abb. 10. Ratte 2931. 28. Tag.

Ratte 2930 (Abb. 9): Am 24. Tage beginnt eine Vergrößerung der Säulenzone, die mit den sekundären Veränderungen als leichte Rachitis angesprochen werden muß.

Ratte 2931 (Abb. 10): Am 28. Tage: Das Bild der Rachitis ist ausgeprägt entwickelt: die gegebene Dosis Vigantol (1 E) hat versagt.

4. Reihe: Mc Collum-Kost und Calcipot D.

Ratte 2847 (Abb. 11) (8. Tag), Ratte 2850 (Abb. 12) (20. Tag) und Ratte 2852 (Abb. 13) (28. Tag): alles normal. Am 8. Tag vielleicht eine leichte Schädigung, die dann bald zurückgeht.

Mc Collum 3143 + 12 mg Calcipot D.

Abb. 11. Ratte 2847. 8. Tag.

Abb. 12. Ratte 2850. 20. Tag.

Abb. 13. Ratte 2852. 28. Tag.

eine Längenzunahme der Säulenzone ein: trotz Vigantol kommt es zur typisch rachitischen Knorpelbildung. Der Befund wird unten diskutiert werden.

4. Calcipot D zeigt genau die umgekehrte Entwicklung: von anfangs hohen, verdächtigen Werten sinkt die Säulenzone auf 85, also den normalen Durchschnittswert.

5. Weizenkeime ($^1/_4$ g) enthalten keine antirachitischen Schutzstoffe, also die in ihnen enthaltenen Wuchsstoffe können nicht als allein wirksam bezeichnet werden (Abb. 14).

6. Weizenkeime mit Calcipot D ergeben von Anfang an niedrige, normal bleibende Werte der Säulenzone. Bereits nach 4 Tagen haben wir den niedrigen Grundwert (Abb. 15).

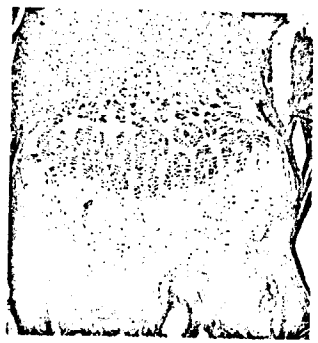

Abb. 14. Ratte 2887. 28. Tag.
McCollum 3143 + $^1/_4$ g Weizenkeime.

Abb. 15. Ratte 2880. 28. Tag.
McCollum 3143 + $^1/_4$ g Weizenkeime + 12 mg Calcipot D.

Was die Trabekelzone betrifft, so zeigt sie durchschnittlich bei den schützenden Zulagen die niedrigsten Werte. Auch hier dürfte eine unvollkommene Resorption Ursache für diese Verlängerungen sein. Die Ursache der Verlängerung der Trabekelzone ist durch die Erdheimsche Analyse in seiner Arbeit „Rachitis und Epithelkörperchen" geklärt worden (s. oben*).

Die für den rachitischen Knochen beweisende verlängerte Knorpelsäulenzone erweist sich demnach nicht als Folge vermehrter Neubildung, sondern vor allem stark verminderter Resorption. Und daraus folgt nun, daß dieser Resorptionsvorgang für das Werden des rachitischen Knochens ausschlaggebend sein muß. Bleibt er schwächer als die Neubildung, so kommt es zu einem gehemmten Ansatz; halten sich beide die Waage, so entsteht das normale Wachstum; wird er stärker, so entstehen atrophische

* Die Zahnuntersuchungen von Euler, auf die bereits verwiesen wurde, erlauben eine noch wesentlich feinere Analyse der Veränderungen.

Vorgänge: die Säulenzone verkürzt sich, wird resorbiert und wir gelangen zu den rein atrophischen Bildern, wie sie in früheren Arbeiten abgebildet wurden (Bd. 153, S. 371; Bd. 189, S. 195; Bd. 167, S. 535).

Entgegen der früheren Anschauung, daß die sogenannten osteoiden Säume entkalkter alter Knochen seien — eine Anschauung, die zu der Verabreichung von Kalkpräparaten bei Rachitis führte —, ist durch die Analysen von M. B. Schmidt, Erdheim und anderen bewiesen, daß osteoide Säume neu angebaute Knochengrundsubstanz sind, die also einer produktiven Tätigkeit der Osteoblasten ihre Entstehung verdanken. Da nun der Knochen selbst einem starken inneren Umbau unterliegt derart, daß der Anbau vorzugsweise von außen und durch Osteoblasten erfolgt, der Abbau vom Innern durch Osteoklasten geleitet wird, entsteht auch im knöchernen Teil des Skelets ein Antagonismus zwischen Anbau und Verbrauch. Für das Verhältnis zwischen diesen beiden Eigenschaften gilt das gleiche wie für die Bildung und Resorption des Knorpels (s. oben). Wir müssen infolgedessen annehmen, daß auch die Osteoklastentätigkeit bei Rachitis erheblicher herabgesetzt ist als die Osteoblastentätigkeit. Das kommt für die Entstehung der Osteomalazie des Erwachsenen in Betracht.

Die Erkenntnis nun, daß Wuchsstoffe für die Bildung des Knorpels wie des Knochens unbedingte Voraussetzung sind, muß zu der Folgerung führen, daß diese bei der Entstehung der krankhaften Gewebe beteiligt sind, sei es, daß sie aus den Diäten stammen, sei es, daß sie aus der Vorversuchszeit im Körper gestapelt sind oder daß der jugendliche Organismus sie zu synthetisieren vermag, der ältere aber nicht mehr. Wir werden unten sehen, daß die letztere Annahme die größere Wahrscheinlichkeit für sich hat. Der Beweis kann natürlich erst geführt werden, wenn chemische Stoffe in genau gemessener Menge zu einer wirklich einwandfreien synthetischen Kost gegeben werden und die Ausscheidung bestimmt werden kann.

Aus der oben angegebenen Versuchsreihe kann man ferner erkennen, daß die Zugabe von Kalk bei experimenteller Rattenrachitis tatsächlich eine Verschlimmerung des Zustandes herbeiführt und so weit führen kann, daß selbst die sonst wirksame Heildosis des Vitamins D nur eine aufschiebende Bedeutung hat, aber keine endgültige prophylaktische.

Im Rahmen dieser Studien ist nun eine Anzahl weiterer Versuche angestellt.

Versuch 71.

In der gleichen Anordnung wie der Versuch 70 wurde die Sherman-Pappenheim-Kost 84 untersucht. Das Ergebnis zeigt bei den Gewichtskurven der jungen Tiere (Tabelle 4), daß sie im allgemeinen sogar eine etwas bessere Zunahme hatten als bei der McCollum-Kost, und daß das ungünstige Verhältnis Phosphor : Kalk schädigend wirkt.

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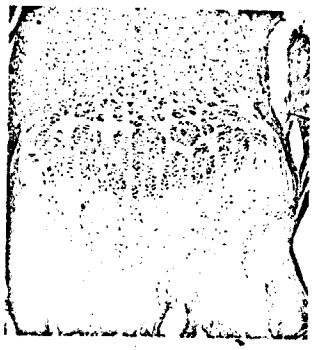

Abb. 14. Ratte 2887. 28. Tag.
McCollum 3143 + $^1/_4$ g Weizenkeime.

Abb. 15. Ratte 2880. 28. Tag.
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In der gleichen Anordnung wie der Versuch 70 wurde die Sherman-Pappenheim-Kost 84 untersucht. Das Ergebnis zeigt bei den Gewichtskurven der jungen Tiere (Tabelle 4), daß sie im allgemeinen sogar eine etwas bessere Zunahme hatten als bei der McCollum-Kost, und daß das ungünstige Verhältnis Phosphor : Kalk schädigend wirkt.

Wenn man aber nun denkt, daß dieser entschieden guten Gewichtszunahme eine Rachitis hätte folgen müssen, so führte dieser Versuch zu

Tabelle 4. Verhalten der Gewichtszunahmen.

Gruppe	4.	8.	12.	16.	20.	24.	28. Tag
Sherman-Pappenheim 84, Original	R 2839	2840	2841	2842	2843	2844	2845
	− 10	+ 3	+ 8	+ 10	+ 10	+ 8	+ 6
Kalksalzgemisch	R 2924	2918	2919	2920	2921	2922	2923
	− 10	+ 3	+ 5	+ 10	+ 15	+ 19	+ 23
Kalksalzgemisch und Vigantol	R 2911	2912	2913	2914	2915	2916	2917
	+ 1	+ 7	+15	+ 7	+ 17	+ 20	+ 25
Calcipot D	R 2832	2833	2834	2835	2836	2837	2838
	− 5	0	+ 5	+ 11	− 10	+ 5	+ 10
1/4 g Keime	R 2867	2868	2869	2870	2871	2872	2873
	− 20	+ 8	+ 5	+ 8	+ 12	+ 15	+ 10
1/4 g Keime Calcipot D	R 2860	2861	2862	2863	2864	2865	2867
	− 15	+ 3	+ 7	+ 13	+ 7	+ 22	+ 20

Tabelle 5. Messung der Wachstumszonen in den Rippenknorpeln. (Werte in μ.)

	Wucherzone	Säulenzone	Trabekel		Wucherzone	Säulenzone	Trabekel
Sherman-Pappenheim 84.				**+ Calcipot D.**			
2839	165	100	335	2832	250	65	200
2840	300	65	200	2833	165	65	165
2841	200	100	250	2834	250	65	200
2842	335	100	250	2835	250	115	250
2843	200	35	85	2836	165	0	0
2844	250	100	115	2837	330	100	165
2845	200	50	100	2838	165	0	0
+ 3,6 mg Kalkgemisch.				**+ Weizenkeime.**			
2924	250	100	335	2867	250	85	165
2918	250	85	165	2868	250	85	200
2919	330	100	165	2869	250	65	165
2920	250	150	330	2870	250	115	165
2921	165	200	330	2871	200	65	165
2922	250	200	250	2872	250	100	165
2923	250	100	250	2873	165	130	165
+ Kalkgemisch + Vigantol.				**+ Calcipot D + Weizenkeime.**			
2911	250	100	250	2860	300	50	250
2912	330	65	330	2861	250	65	200
2913	200	100	250	2862	250	50	165
2914	250	150	200	2863	250	100	250
2915	300	50	200	2864	165	130	165
2916	200	100	200	2865	165	100	165
2917	250	115	165	2866	200	50	130
Norm!							
Diät X + 1/2 g Hefe.				**Diät X + 1 g Hefe.**			
2888	415	65	250	2890	415	100	250
2889	250	85	250	2891	330	100	330

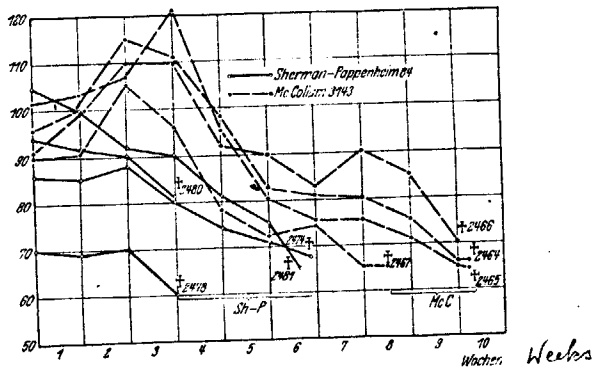
einer vollen Enttäuschung, denn aus der Tabelle 5 läßt sich erkennen, daß die Knorpelsäulenzone überall niedrig geblieben ist und keine Rachitis sich entwickelt hat*.

Beide Kostformen sind also in bezug auf den Wachstumsvorgang völlig verschiedenartig. Es läßt sich noch nicht erkennen, ob hier eine Veränderung des Produktions- oder des Resorptionsvorganges eingesetzt hat. Dazu sind weitere Untersuchungen erforderlich. Die Sherman-Pappenheim-Kost 84 kann nicht als sicher rachitogen bezeichnet werden. Unbekannte Zusatzfaktoren sind zu untersuchen.

Versuch 72a.

Versuche mit älteren Ratten (Anfangsgewicht 70—105 g).

Die Sherman-Pappenheim-Kost 84 wurde in gleicher Form wie in Versuch 71 benutzt.



Kurve 1.

Die McCollum-Kost mußte modifiziert werden, weil Weizengluten nicht zu erhalten war. Statt dessen wurde gereinigtes Casein gegeben. Außerdem wurden statt Grieß die ganzen Weizen- bzw. Maiskörner gegeben (s. oben). Diese Kost wird oft als McCollum-Kost angeführt mit Ausnahme der Caseinverwendung.

In Kurve 1 sieht man nun, daß diese älteren Ratten gewichtsmäßig ganz anders als junge reagieren.

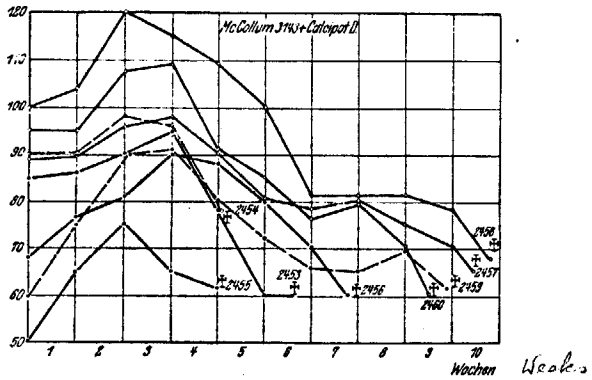
Sherman-Pappenheim-Kost: Statt der bei jüngeren Ratten noch eintretenden Gewichtszunahme (s. Tabelle 4) finden wir von Anfang an eine fortschreitende Abnahme bis zum Tod (3½—6 Wochen).

* Auch hier ergeben die Zahnuntersuchungen Eulers weitere Aufschlüsse.

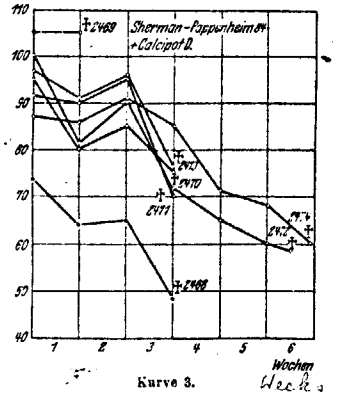
Bei McCollum-Kost finden wir anfangs bis zu 3 Wochen einen ziemlich starken Gewichtsanstieg, dann schnellen Abfall und Tod in 8—10 Wochen.

Versuch 72b.

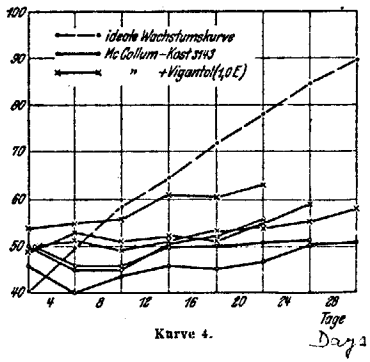
In diesen Versuchen wurden zu den beiden genannten Kostformen täglich 12 mg Calcipot D gegeben. Aus Kurve 2 (McCollum-Kost)



Kurve 2.



Kurve 3.



Kurve 4.

und Kurve 3 (Sherman-Pappenheim-Kost) ergibt sich, daß das nichts an dem Verlauf der zum Tod unter Gewichtsabfall führenden Kurven ändert.

Histologisch zeigen sämtliche Ratten dieser Versuchsreihen reine Atrophien. Es fehlen auch Zeichen einer während des Versuchs überstandenen Rachitis*.

Ursächlich kommen als fehlend in Betracht: Wuchsstoffe, Aminosäuren, Mineralien. Vielleicht enthält Weizenkleber derartige Stoffe, z. B. einen bisher noch unbekannten Faktor, der die Bildung von Knochengrundsubstanz anregt? Das ist in weiteren Versuchen zu entscheiden.

Vergleichsweise sei die Normalgewichtskurve bei Vollkost mit der Gewichtszunahme bei McCollum-Kost 3143 in Kurve 4 gegenübergestellt.

Beurteilung von Versuch 72a und b.

Zunächst ist man geneigt, den verschiedenen Verlauf der Gewichtskurven bei der Wirkung der Rachitisdiäten in diesen beiden Versuchsabteilungen auf die Modifikation der McCollum-Kost zurückzuführen. Doch ist das ziemlich unwahrscheinlich, weil ja auch die Sherman-Pappenheim-Kost den gleichen Gewichtsabfall zeigt, obwohl sie nicht modifiziert wurde. Infolgedessen dürfte die Annahme die größere Wahrscheinlichkeit haben, daß das höhere Alter der Tiere und die damit steigenden Ansprüche des reiferen Tieres die endogene Ursache der verschiedenartigen Reaktion sind. Da nun außerdem die Körnerzulage wohl zweifellos wesentlich vollkommener sein muß als die Verwendung von Grieß, so müßte man eher eine Verbesserung der Wirkung erwarten. Der Wandel von Weizengluten in Casein kann immerhin als Ursache in Betracht kommen. Die Versuche werden weiter fortgesetzt.

Die größere Wahrscheinlichkeit liegt darin, daß die älteren Ratten infolge ihrer höheren Ansprüche die umfassenden Mängel der Rachitisformen deutlicher hervortreten lassen, als bei den jüngeren Ratten möglich war. Am wahrscheinlichsten ist bei den jüngeren Ratten die aus der Vorfütterung stammende Mitwirkung von Reserve-Wuchsstoffen oder deren Produktion in geringer Menge.

12 mg Calcipot D verbessern hier nichts. Ob höhere Zulagen eine Verbesserung erreichen, konnte noch nicht untersucht werden. Auf Grund der früheren Erfahrungen aber möchte man der Erwartung Ausdruck geben, daß für die Wirkung beim älteren Individuum eine wesentliche Erhöhung der Hefekomponente erforderlich sein dürfte. Wuchsstoffe haben offenbar nicht jene Spurenwirkung wie Vitamin D.

Diskussion der Ergebnisse.

1. Beide untersuchten Rachitisdiäten lassen erkennen, daß sie auch sonst äußerst unvollkommen sind, besonders die Sherman-

* Die Zahnuntersuchungen Eulers ergaben wieder weitere Aufschlüsse.

Pappenheim-Kost; gegenüber der Norm bleiben die Ratten doch weit im Gewicht zurück, ältere Ratten nehmen sogar stark ab, entwickeln eine Atrophie hohen Grades und sterben.

Daraus ist zu folgern, daß die Rachitisdiäten nicht nur einen Vitamin D-Mangel aufweisen müssen, sondern auch noch andere Mängel. Auf diese ist schon oft aufmerksam gemacht. Ich verweise auf die Zusammenstellung von Bomskov. Die aus diesen Mängeln entstehenden Umwelteinwirkungen sind der eine, nahrungsbedingte Teil des prärachitischen Zustandes.

2. Ältere Ratten entwickeln aber bei einer leicht modifizierten Mc Collum-Kost und bei unveränderter Sherman-Pappenheim-Kost eine Atrophie, wo jugendliche noch Rachitis bekommen. Aus dem Unterschied zwischen diesen beiden Reaktionen der Lebensalter wird man vielleicht dazu gelangen können, die zweite endogene Komponente des prärachitischen Zustandes erforschen zu können.

3. Reines Vitamin D besitzt nur eine aufschiebende prophylaktische Wirkung, wenn zu viel Kalkgemisch verabreicht wird. Hier wird man notwendigerweise die entwicklungsgeschichtlichen tieferen Ursachen der Rachitisbereitschaft zu untersuchen haben. Wahrscheinlich werden hier vergleichende Untersuchungen in der absteigenden Tierreihe bis zu den Knorpelfischen notwendig sein, um chemisch die Relation Phosphor zu Kalk, andererseits die Beziehung zum Vitamin D zu erforschen. Diese Vorgänge dürften mit Sicherheit aufs engste an die Entwicklung der Hormondrüsen geknüpft sein.

4. Die histologische Untersuchung der Wachstumszonen erlaubt es, die feineren Vorgänge zu studieren. Hier sei erwähnt, daß ebenso wie in der VII. Mitteilung (S. 228) bei diesen Präparaten die Störungen der vaskulären Resorption auffielen: Blutarmut der Kapillaren, oft fast völliges Zusammenfallen kamen häufig vor. Auf Grund der früher erhobenen Befunde darf angenommen werden, daß hier ein zusätzlicher Vitamin A-Mangel beteiligt sein könnte. Und es ist nicht unwahrscheinlich, daß die erhöhte Infektionsbereitschaft des Rachitikers mit diesen gestörten Kapillarfunktionen und deren Folgen zusammenhängen könnte.

5. Ferner ist zu untersuchen, ob eine der Mesotrophie ähnliche Lebensmöglichkeit an der untersten Grenze etwa zur Erklärung des prärachitischen Zustandes ausreichen könnte.

6. Praktisch läßt sich aus diesen Versuchen bereits schließen, daß die isolierte Verabfolgung von Vitamin D ausreichend prophylaktisch zu wirken vermag. Doch vermag diese Prophylaxe nicht jene Schäden zu beseitigen, die Folge der übrigen, oben nachgewiesenen Mängel der Kost sind, welche zur Prärachitis führen. Dazu sind Kombinationspräparate entschieden aussichtsreicher.

7. Die bisherige experimentelle Rachitisforschung ist demnach nur geeignet, das Vitamin D zu standardisieren. Der Gesamtumfang „Rachitis“

sowie die Erklärung der ihr ähnlichen Krankheiten muß durch eine wesentliche Erweiterung der Forschungsmethoden versucht werden. Auch die bisherigen Testverfahren reichen nicht aus, da sie nicht fein genug sind, die feineren Teilphasen des Wachstumsvorganges erkennen zu lassen.

Die Rachitisversuche müssen stets durch die Berücksichtigung der Zahn- und Kieferveränderungen vervollkommnet werden. Auf die Untersuchungen von Euler wird ausdrücklich verwiesen (s. 18. Mitt.).

Wir müssen uns von der bisherigen einseitigen Vitamin D-Forschung freimachen und einer umfassenderen eigentlichen Rachitisforschung zuwenden. Der Weg dazu wurde in der 8. und 9. Mitteilung gezeigt. Die Bildung der Antikörper bei der mesotrophischen Kost wird in der nächsten Mitteilung beschrieben.

(Abgeschlossen am 5. Januar 1942.)

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Nogyo Gijutsu Kenkyusho Hokoku G (Bulletin of the National Institute of Agricultural Science (Japan)), p. 21-27.

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On the Effect of Calcium Citrate upon Calcium metabolism.

By Tsuyoshi Nakajima and Junichi Otsuka

(For a brief English synopsis, refer to the original text on p. 27.)

Introduction

As one of the nutritional deficiencies of cattle raised in our country, the lack of calcium in-take can be mentioned. We have conducted a study on this for a long time. In view of the fact that the effect of calcium-deficient feed upon the animal bodies is large, we have observed a keen necessity of adding calcium to animal feed. Even nowadays, calcium is believed to be lacking in many animal feeds that the Japanese farmers used for their cattle.

The problem of calcium deficiency should cease as soon as animal feed containing a large amount of calcium starts being supplied in large quantities. In our country, however, the feed sources for cattle raising are limited in variety. Economically, too, it is highly difficult to supply a large amount of animal feed containing calcium, with a result that its supply has been derived from the so-called calcium agents. In this case, calcium carbonate has been used in large amounts as a calcium agent in the past.

Steenbock (1) et al. carried out a test on white mice by using calcium lactate, calcium carbonate, calcium phosphate and calcium sulfate, etc. and reported that there was no difference in effect amongst them as calcium agents. Iizuka (2) reported that it is sufficient if the animal feed lacking in calcium is supplemented with calcium carbonate. Hara (3) reported that the rate of calcium utilization in calcium carbonate was 62 per cent as compared with milk. In order for calcium salts to be utilized as a calcium source in the animal bodies, it is necessary for the same to be dissolved in the digestive pipes. However, calcium carbonate has a defect of its solubility being low.

Osteo malacia whose occurrences in our country are large and which is be-

lieved to stem from the deficiency of calcium in animal feed is a disease which is accompanied by the excess discharge of phosphorus and the removal of ash from the bone. The excess discharge of phosphorus appears along with acidosis. In view of the fact that the reason for this disease is considered to be giving feed mainly containing cereals to the animals, this disease is believed to be accompanied by acidosis.

For the treatment of this disease, therefore, it is necessary to prevent the development of acidosis and it is necessary to supply fixed bases which are consumed in large quantities in the case of acidosis. Albright (4) reported that, in such a disease as this, the production of ammonia is obstructed, with a result that the consumption of fixed bases becomes all the more marked. Therefore, chemicals which are used in the treatment of this disease were those which work on the living bodies as alkalis. In order to reduce the excretion of calcium, it is necessary for the same to contain sodium or potassium or calcium.

In any case, therefore, if it is desired to have a calcium agent which is better than those which are currently used, it has to be a compound which is satisfactorily dissolved in the digestive pipes, which is absorbed satisfactorily and which serves as an alkali on the living bodies. The calcium salts of organic acids can be considered as those meeting these requirements.

We used calcium citrate and examined the effect of the same upon the bone compositions of white rats (albinos) and rabbits and upon calcium metabolism.

Testing Method

The test was divided into two parts. One part comprised white rats on which the effect of sodium citrate and calcium citrate was observed for a comparatively long period of time. The other part comprised domesticated rabbits in which the function mechanism of these salts was observed on the basis of the metabolism test on calcium and phosphorus.

In the test using white rats (albinos), first, the young mice coming from the same mother were classified into three groups and they were given such feed as are described in Table 1 for a period of 60 days. The white rats were separated one by one to be placed in a metal net cage, they were allowed to freely drink water and eat feed. Three times a week, they were weighed immediately before feeding. The test result was analyzed after examining the change in the bones. Since the method used was the same as the method which was used in our previous report (5), its description will be omitted from this report.

In the test employing domesticated rabbits, unless indicated each time, the grown-up rabbits whose weight was approximately two kilograms were subjected to a preliminary test for a period of three days and a main test for a period of three days. The amounts of calcium and phosphorus taken in and discharged were measured, and the results obtained were compared.

The method of feeding was the same as in the case of white rats. Urine and feces were collected at one time during the period of three days. Urine was used as it was for the determination of calcium and phosphorus. The feces were converted into "ash" before determination. The Clark-Collip method (6) was used in the analysis of calcium and the method of Shioiro (7) was used for the analysis of phosphorus.

The basic feed for the test was the feed prepared by mixing the same amounts of rice-bran and wheat-bran. Its composition was 12.95 per cent of water, 8.09 per cent of crude ash, 0.08 per cent of calcium and 2.62 per cent of phosphorus, with the ratio between phosphorus and calcium being extremely dissatisfactory. The test was carried out by adding additives to this basic feed in such a manner that recording can be made for each item.

(Table 1 Feed Components.)

Experimental Result and Observations

1. Effects of Sodium Citrate and Calcium Citrate upon the Bones of White Rats (Albinos).

The white rats (albinos) which were used in the test were classified into three groups and these were given the respective types of feed. The body growths observed are shown in Figure 1. It is seen that the growth of Group 2 was the best and this was followed by Group 3 and Group 1. In Group 2, the body weight increased continuously. Group 3 showed a tendency for the body weight to increase at the beginning of the test, with the weight being just maintained after the middle of the test period. Group 1 showed an increase as in Group 2 for the first 10 days, showing a tendency for the weight to drop thereafter.

(chart 1 Increase in body weight of female rats reared with three different diets.)

As a result of the above, it was learned that calcium citrate gives a better effect upon the growth of the bodies than calcium carbonate. Even in the case of sodium citrate, there was observed an increase in the body weight at the start of the test period despite the fact that the ratio between phosphorus and calcium was dissatisfactory in the feed (unlike in the case of unhulled rice) (5). It is believed, therefore, that it brought about a favorable effect upon the maintenance of calcium in the body.

After the completion of the test, these white rats (albinos) were killed and their bones were examined. Even in Group 1, those bead-like knots, etc. which were observed in the bones of white rats (albinos) which we raised with unhulled rice alone (5, 8), were not observed. In groups 2 and 3, they were, of course, not observed.

The bone compositions of the white rats belonging to these three groups are shown in Table 2. There were increases in all components (except for

water in all three groups as compared with the bone compositions in the test result we obtained by raising them with unhulled rice (5). Especially noteworthy was Group 2, which showed a marked increase. Despite the fact that they were raised with feed containing the same calcium amount as unhulled rice, it was possible to form bones which are richer in calcium and dry products than in Group 3.

In addition, calcium occupies 19 per cent in the dry bone in Group 1. In the case of white rats (albinos) which were raised with feed consisting of unhulled rice, it was less than 16 per cent. Despite the fact that the ratio between phosphorus and calcium was dissatisfactory and the contents were small in the feed used, the calcium portion was found to be larger.

(Table 2 Composition of Bones.)

It was learned from the above results that sodium citrate has the effect of holding calcium in the body and, even though the ratio between phosphorus and calcium in the feed may be dissatisfactory, and even when their contents are smaller than those which were considered to be necessary in the past, it has the effect of at least preventing the change in the bones.

This is mainly because of the existence of sodium. It is believed that it is due to the fact that the consumption of calcium by the excess discharge of phosphoric acid is replaced by sodium. It is assumed that citric acid serves in the prevention of acidosis.

Calcium citrate showed a better effect upon the development of the body and the accumulation of calcium in the body than calcium carbonate. We reported (5) that no favorable effect would be brought about on the growth of the body by merely ameliorating the ratio between phosphorus and calcium in the feed by simply adding calcium carbonate to unhulled rice and that the body weight would not increase unless phosphorus, too, is supplied at the same time to bring the amounts of these two to the required levels.

In the current experiment, calcium was supplemented. However, the amount of its supplement was smaller than the required amount. Nevertheless, the body weight increased and a result which was somewhat different from the previous result was obtained. This is because of the fact that the absorption of calcium citrate is better than that of calcium carbonate. Regarding the required amount which has been considered in the past, it seems that the amount changes each time, depending upon the compound which is supplemented.

2. Effect of Sodium Citrate and Calcium Citrate upon the Metabolism of Calcium and Phosphorus in Domesticated Rabbits,

If the rabbits were raised with feed prepared by mixing the same amounts of rice bran and wheat bran (which diet will hereafter be called bran diet), the so-called osteomalacia would develop, according to Iizuka (9). Iizuka reported that it is necessary to add calcium carbonate to animal feed in order to prevent or treat the same.

At first, we carried out a test on the difference between the metabolism of calcium and phosphorus in domesticated rabbits which were raised with this feed and the metabolism of calcium and phosphorus as obtained in the regular case. The result obtained is shown in Chart 2.

(Chart 2 Metabolism of Ca and P in Rabbits reared with Bran Diet.)

In the case of both phosphorus and calcium, the amounts discharged were greater than the amounts taken in, with the amounts of discharge being greater toward the end of the test period. When an observation is made in urine, there was observed a gradual increase in the amount of phosphorus discharged as the number of test days increased. In the case of calcium, however, the situation was different from this result and there was observed a sharp rise, with the amount of its discharge being almost the same as the amount of the discharge of phosphorus toward the end of the test period.

The fact that sodium and potassium are discharged on a priority basis as

compared with calcium in a fixed base in the living body was observed in connection with hunger-afflicted acidosis. In the current test, sodium and potassium were discharged at first and, as the number of test days increased, the amount of calcium discharged increased. The rabbits suffered from acidosis by the cereal-rich feed and this may be a phenomenon which accompanied the buffer action of the phosphate system.

We learned in Experiment 1 that sodium citrate has the effect of keeping calcium in the living body and it was confirmed that calcium citrate has the effect of accumulating calcium in the body to a greater extent than calcium carbonate. An attempt was made to clarify the mechanism for showing this effect by using the test animals which showed an excessive amount of calcium discharged as obtained in this experiment.

I. Effect of Sodium Salts upon the Metabolism of Calcium and Phosphorus.

Table 3 shows the effect upon the metabolism of calcium and phosphorus of domesticated rabbits which were reared with feed which was prepared by adding sodium citrate or sodium carbonate at the rate of 10 per cent to bran diet.

(Table 3 Metabolism of calcium and phosphorus in male rabbits reared with bran diet supplemented with sodium citrate or sodium carbonate.)

In the case of Group 1 in which sodium citrate was added, there were observed an excessive discharge of phosphorus and the equilibrium of calcium, and it was possible to prevent the excess discharge of calcium with sodium citrate.

In the case of Group 2 in which sodium carbonate was added, there was an excessive discharge of both phosphorus and calcium in the total of feces and urine. However, the amount of calcium discharged in urine was reduced to a marked extent. Thus, it was seen that the discharge of calcium was reduced by the effect of sodium ions which were common to the compounds which were added.

The reason why there was a difference in the amount of calcium discharged in feces between the two groups lies in the fact that, both sodium citrate and sodium carbonate are compounds with a high degree of solubility, that there is a possibility for the calcium which has been freed in the intestines to form calcium citrate with the former and calcium carbonate with the latter and that the said difference was produced because of the higher solubility of the former and the lower solubility of the latter.

II. Effect of Citric Acid upon the Metabolism of Calcium and Phosphorus

In this test, domesticated rabbits were reared with bran diet for a period of six days and then the average daily amounts of calcium and phosphorus taken in and discharged were measured for a period of three days. For a period of six days thereafter, they were reared with bran diet supplemented with 10 per cent of citric acid. Likewise, for a period of three days, the amounts of metabolism were measured and compared.

The result obtained is shown in Table 4. It was possible to somewhat prevent the amount of phosphorus discharged by adding citric acid to bran diet.

This is believed to be due to the fact that citric acid serves as an alkali by the perfect combustion in the living body, with a result that it has a slight effect of preventing acidosis and that the amount of phosphorus discharged is reduced.

(Table 4 Metabolism of calcium and phosphorus in rabbits reared with bran diet supplemented with citric acid.)

III. Effect of Calcium Citrate and Calcium Carbonate upon the Metabolism of Calcium and Phosphorus.

From the above-described experiment, it was assumed that there would be a difference in accumulation and absorption when calcium citrate was used for the purpose of giving calcium. Therefore, the following experiment was carried out:

First, four rabbits each were killed on the sixth or 10th day after the

start of the test for the main purpose of examining the absorption of calcium. The alimentary canal was removed and the contents of the digestive pipe were cleaned with water. These were converted into ash and calcium and phosphorus were determined. At the same time, a day's portion of feces and urine were collected and the amount of the calcium and phosphorus metabolism was measured.

As for the feed used, 7.5 grams of calcium citrate was added to the 100 grams of the bran diet in Group 1 and four grams of calcium carbonate was added to 100 grams of the bran diet in Group 2. Both groups were reared with feed whose ratio between phosphorus and calcium was adjusted to 1.5.

The result of the test obtained was shown in Table 5. In both groups, calcium and phosphorus were accumulated in the body. However, a greater amount was accumulated in Group 1. The amount of calcium which is left in the digestive pipe was smaller in Group 1 than in Group 2. Therefore, both the absorption and accumulation of calcium were greater in Group 1.

(Table 5 Metabolism of calcium and phosphorus in male rabbits reared with bran diet supplemented with calcium citrate and calcium carbonate.)

Next, the effect of both compounds upon the rabbits afflicted with the so-called osteomalacia was tested. Between the domesticated rabbits which were reared for a long period of time with feed which is deficient in calcium and the domesticated rabbits with a greater amount of calcium accumulated in the body through normal feeding, there was observed a difference in the amount of calcium absorbed even when calcium carbonate is given and a greater amount is accumulated in the former, too.

Therefore, after the rabbits were reared with bran diet for a period of 60 days, thereby causing them to be afflicted with osteomalacia, calcium citrate and calcium carbonate were added in the same manner as in the previous test and they were separated into Group 1 and Group 2. A six-day treatment test was carried out and the average daily amounts of calcium and phosphorus taken in and discharged in a period of three days thereafter were

measured and the results obtained were compared.

The test result obtained is shown in Table 6. During the period of six days of the test period, the body weight was found to have incrased in both groups. In Group 1, both calcium and phosphorus showed an accumulation. In Group 2, calcium was accumulated but there still was an excessive amount of phosphorus discharged.

(Table 6 Metabolism of calcium and phosphorus in male rabbits reared with bran diet supplemented with calcium citrate or calcium carbonate after being reared with bran diet for a period of 60 days).

An examination of their amounts discharged in urine showed that Group 1 was smaller than Group 2 insofar as calcium was concerned, thereby indicating the fact that calcium citrate is better than calcium carbonate for the accumulation of calcium in the body.

From the above-described test result, it can be stated that calcium citrate is a better calcium agent than the calcium carbonate which is widely used. In the case where it is assumed that acidosis has been induced because of the use of feed which is excessively rich in cereals, it seems necessary to use the sodium salts or calcium salts of organic acids as calcium agents to be added to animal feed.

Conclusion:

(1) By using white rats (albinos), the effect of citric acid, sodium citrate and calcium citrate upon the metabolism of calcium and phosphorus was tested.

(2) Sodium ions have the effect of reducing the discharge of calcium and remedying the effect that the feed which is excessively rich in cereals has upon the metabolism of calcium.

(3) Calcium citrate increases the absorption and accumulation of calcium to a greater extent than calcium carbonate. It is a calcium agent which is especially effective in the prevention and treatment of osteomalacia.

At the end, the authors would like to offer sincere thanks to Dr. Nagahata,

Director of the Physiological Department of this Institute who was kind enough to read the manuscript.

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Translated by Carl Demrick Associates, Inc./RO/db

Table 1. Feed Components

Group	Basal Feed	Supplemented Chemicals			Feed Constitution		
	Unpolished Rice	Sodium Citrate	Calcium Citrate	Calcium Carbonate	Calcium	Phosphorus	Ca : P
1	93.5g	1.5	— g	— g	0.02%	0.20%	1 : 10
2	93.5	—	1.5	—	0.23	0.20	1 : 0.8
3	100.0	—	—	1.5	0.23	0.20	1 : 0.7

Table 2. Composition of Bone

Group	Sex	Number of Animals	Analysis of Fresh Bone			Analysis of Dry Bone		
			Dry Matt.	Water	Ash	Ash	Calcium	Phosphorus
1	♀	4	50.62%	49.33%	22.06%	43.77%	19.15%	7.64%
2	♀	4	61.29	33.71	32.24	52.65	22.61	9.03
3	♀	3	53.46	41.54	29.69	50.81	19.53	8.87

Table 3. Metabolism of Calcium and Phosphorus in Male Rabbits Reared with Bran Diet Supplemented with Sodium Citrate or Sodium Carbonate

Dietary Supplement	Number of Animals	Analysed Element	Intake (1)	Excreta (2)	Balance (1)-(2)	Proportion of Excreta to Intake			
						Intake	Excreta	Urine	Feces
Sodium Citrate	4	Calcium	49mg	49mg	0mg	100	100	33	67
		Phosphorus	732	1110	−378	100	152	70	82
Sodium Carbonate	4	Calcium	23	456	−433	100	198	10	188
		Phosphorus	344	474	−130	100	137	86	51

Table 4. Metabolism of Calcium and Phosphorus in Rabbits Reared with Bran Diet Supplemented with Citric Acid

Dietary Supplement	Analysed Element	Intake (1)	Excreta (2)	Balance (1)-(2)	Proportion of Excreta to Intake			
					Intake	Excreta	Urine	Feces
None	Calcium	41mg	187mg	−146mg	100	456	10	90
	Phosphorus	632	744	−112	100	117	55	45
Citric Acid	Calcium	44	238	−194	100	543	2	93
	Phosphorus	673	750	− 77	100	111	43	57

Note : The same five male rabbits were employed in two kinds of the experiments.

Table 5. Metabolism of Calcium and Phosphorus in Male Rabbits Reared with Bran Diet Supplemented with Calcium Citrate or Calcium Carbonate

Dietary Supplement	Number of Animals	Length of Period	Analysed Element	Intake (1)	Excreta (2)	Balance (1)-(2)	Residue in Intestine	Proportion of Excreta to Intake: Intake	Excreta	Urine	Feces
		Days		mg	mg	mg	mg				
Calcium Citrate	4	6	Calcium	1114	379	+735	674	100	34	2	32
			Phosphorus	1709	1031	+627	1032	100	63	6	57
	4	10	Calcium	1194	786	+408	899	100	66	1	65
			Phosphorus	1830	1066	+764	1172	100	58	3	55
Calcium Carbonate	4	6	Calcium	1357	1283	+ 74	783	100	95	16	79
			Phosphorus	2018	1397	+621	1751	100	69	7	62
	4	10	Calcium	1357	1165	+192	1795	100	86	7	79
			Phosphorus	2018	1258	+760	2312	100	62	9	53

Table 6. Metabolism of Calcium and Phosphorus in Male Rabbits Reared with Bran Diet Supplemented with Calcium Citrate or Calcium Carbonate after Reared with Bran Diet for 60 Days

Dietary Supplement	Number of Animals	Length of Period	Analysed Element	Intake (1)	Excreta (2)	Balance (1)-(2)	Proportion of Excreta to Intake: Intake	Excreta	Urine	Feces
		Days		mg	mg	mg				
Calcium Citrate	5	6	Calcium	1285	920	+365	100	72	6	66
			Phosphorus	1276	1243	+ 33	100	97	15	82
Calcium Carbonate	5	6	Calcium	919	853	+ 66	100	93	18	75
			Phosphorus	1308	1328	− 20	100	102	12	90

Chart 1. Increase in Body Weight of Female Rats Reared with Three Different Diets

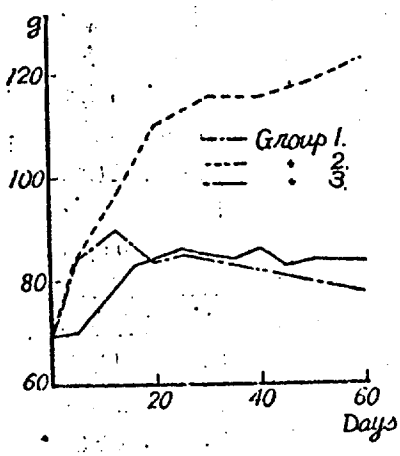
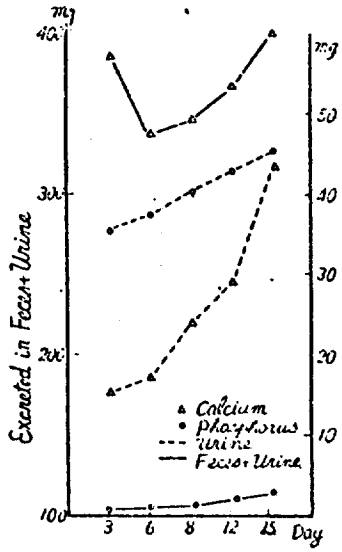


Chart 2. Metabolism of Ca and P in Rabbits Reared with Bran Diet



Note: Assuming that the intake of Ca and P is 100mg each.

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クエン酸カルシウムのカルシウム代謝に及ぼす影響について

中島　剛・大塚閏一

緒　言

我が国の家畜の栄養上の欠陥の一つとしてカルシウム攝取量の不足が挙げられ，我々もこれに関して従来から研究を行い，カルシウム不足飼料が動物体に及ぼす影響の甚大なことからカルシウムを飼料に添加する必要を痛感しているが，未だ一般農家の家畜の飼料中にはこれが不足しているものが多い。このカルシウム不足はカルシウムを多含する飼料を豊富に給することにより解消する筈であるが，我が国の家畜の飼料源は割合に種類が少く，経済的にもカルシウムを多く含む飼料を多給するには困難が多く，カルシウム剤と称されるものにこの給源を求めなければならないのが普通である。この必要性からカルシウム剤として従来多く用いられているのは炭酸カルシウムである。

STEENBOCK[1] 等は乳酸カルシウム，炭酸カルシウム，燐酸カルシウム，硫酸カルシウム等を用い，白鼠で試験を行い，カルシウム剤としては効果に差異がなかつたと報告し，飯塚[2] はカルシウム不足飼料にはカルシウムを炭酸カルシウムで加えば充分であると報告している。原[3] は炭酸カルシウムのカルシウム利用率は牛乳に比較して62%であつたと報告している。カルシウム塩類がカルシウム源として動物体に利用されるためにはそれが消化管内で溶解されることが必要であるが炭酸カルシウムは溶解度が低い欠点を持つている。

又我が国に多発し主として飼料中に含有されるカルシウムの欠乏が原因とされている骨軟症は燐の排泄過剰と共に骨の脱灰現象を呈する疾病である。燐の排泄過剰はアシドーシスと併行して現れるもので穀類偏重飼料がこの疾病の原因となつていることからも，この疾病にはアシドーシスを伴つていることが考えられる。故にこの疾病の治療にはアシドーシスを防止することが必要でアシドーシスの場合に多く消耗される固定塩基の補給が必要である。ALBRIGHT[4] はこの様な疾病では腎臓に於けるアンモニヤの生産が障碍されて固定塩基の消耗が一層甚しくなることを報告している。故にこれが治療に用いる薬剤は生体にアルカリとして作用するものでカルシウムの排泄を節約するためにナトリウム又は加里を含み或はカルシウムを含んでいることが必要である。

故にいずれの場合にも飼料に添加するカルシウム剤に現在使用されているものより尚良好なものを望むならば，消化管内でよく溶解し吸収が良好で，生体に対してアルカリとして作用する化合物でなければならない。この目的に合致するものとして有機酸のカルシウム塩類が考えられるので，我々はクエン酸カルシウムを用い白鼠及び家兎の骨成分及びカルシウム代謝に及ぼす影響を試験した。

試験方法

試験は2部に分け，1部は白鼠でクエン酸ソーダ及びクエン酸カルシウムの影響を比較的長時間観察したもので，他の1部は家兎でカルシウムと燐の代謝試験からこの塩類の作用機転について考察したものである。

先ず白鼠を使用した試験では同腹仔を3群に分け，第1表の如き飼料で60日間飼育した。白鼠は一匹宛分離して金網籠に入れ，飲水及び飼料は自由に攝取させ，1週間に3回給飼直前に体重を測定した。試験結果は骨の変化を調べてから分析して比較したがその方法は我々[5] の前の報告と同様であるから省略する。

次に家兎を用いた試験では体重約 2kg 前後の成兎を特にその都度記載した以外は予備試験3日間，本試験3日間とし，カルシウム及び燐につい

Table 1. Feed Components

Group	Basal Feed: Unpolished Rice	Supplemented Chemicals: Sodium Citrate	Calcium Citrate	Calcium Carbonate	Feed Constitution: Calcium	Phosphorus	Ca : P
1	93.5g	1.5g	— g	— g	0.02%	0.20%	1 : 10
2	93.5	—	1.5	—	0.25	0.20	1 : 0.8
3	100.0	—	—	1.5	0.23	0.20	1 : 0.7

て摂取及び排泄量を測定して，その結果を比較したものである。飼育方法は白鼠の場合と同様である。尿と糞は3日分をまとめて採集し，尿はそのままでカルシウムと燐の定量に供し，糞は灰化してから定量した。カルシウムの分析は CLARK-COLLIP 法[6]を燐は塩入[7]の方法を用いた。試験の基礎飼料は米糠と小麦麩の等量を混合したもので，成分は水分12.95%，粗灰分8.09%，カルシウム0.08%及び燐2.62%で燐とカルシウムの比率は極めて悪いものである。この基礎飼料に試験項目毎にその都度記載する様な添加物を加えて試験を行つた。

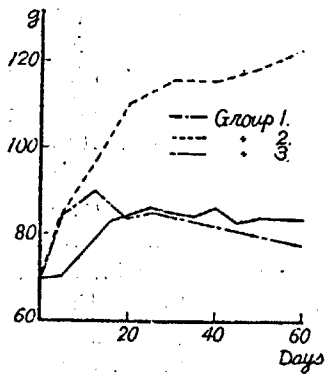
実験成績及び考察

1. クエン酸ソーダ及びクエン酸カルシウムが白鼠の骨に及ぼす影響

試験に用いた白鼠は3群に分け，夫々の飼料で飼育した結果その発育は第1図の如くで，第2群の発育が一番良く，第3，1群とこれに続いている。第2群は終始体重は上昇し，第3群は試験初期は上昇の傾向を示し，中期以降体重を維持するに止まり，第1群は初期約10日間第2群と同様に上昇し爾後下向の傾向を示した。

この結果はクエン酸カルシウムが炭酸カルシウムよりも身体の発育に好い影響を与えることが分り，又クエン酸ソーダでも玄米だけの場合[5]と異つて飼料中の燐とカルシウムの比率が悪いのに試験初期に体重の上昇を示すことからカルシウムの体内保持に好い影響を齎したのだと思われる。

Chart 1. Increase in Body Weight of Female Rats Reared with Three Different Diets



試験終了後これらの白鼠を殺してその骨を見れば，第1群でも我々[5][8]が玄米だけで飼育した白鼠の骨に認められた念珠状結節等の変化は認められず，第2，3群では勿論認められなかつた。

3群の白鼠の骨成分は第2表の如くで，我々[5]が玄米のみで飼育した試験の結果の骨成分よりも3群共に水分を除いて何れも増加した。特に第2群が一番多く玄米と同じカルシウム分を持つた飼料で飼育したにも拘らず，第3群よりも乾物量，カルシウムに富んだ骨を形成させることが出来た。又第1群ではカルシウムが乾骨中に19%を占めているが，玄米のみの飼料で飼育した白鼠では16%よりなく，燐とカルシウムの比率も悪く，その含量も少ない飼料であるのにカルシウムが増加していた。

Table 2. Composition of Bone

Group	Sex	Number of Animals	Analysis of Fresh Bone: Dry Matt.	Water	Ash	Analysis of Dry Bone: Ash	Calcium	Phosphorus
1	♀	4	50.62%	49.33%	22.06%	43.77%	19.15%	7.64%
2	♀	4	61.29	33.71	32.24	52.65	22.61	9.63
3	♀	3	53.46	41.54	29.69	50.81	19.53	8.87

以上の結果からクエン酸ソーダはカルシウムを身体に保持させる効果があり，飼料中の燐とカルシウムの比率が悪く，又それらの含量が従来から唱えられている必要量よりも少くても骨の変化を防止する作用のあることが分つた。これは主としてナトリウムの存在によるもので，燐酸の排泄過剰によるカルシウムの消耗をナトリウムが代替しているからと思われ又クエン酸がアシドーシスの防止に役立つているからであろうと考えられる。

炭酸カルシウムよりもクエン酸カルシウムの方がカルシウムの体内蓄積と身体の発育に好い影響を現した。我々[8]は玄米に炭酸カルシウムを加えて飼料中の燐とカルシウムの比率を好くしただけでは身体の発育に好い影響を得られず，燐も同時に補つて飼料中の両者を必要量にしなければ体重は上昇しないと報告した。今回の試験ではカルシウムを補つたとはいえ必要量から見れば少かつたが体重は上昇し前の成績とは稍異つた結果を得た。これはクエン酸カルシウムの方が炭酸カルシウムよりも吸収が好いためで，従来から考えられている必要量については添加する化合物によつて其の都度変更を要するのではないかと思われる。

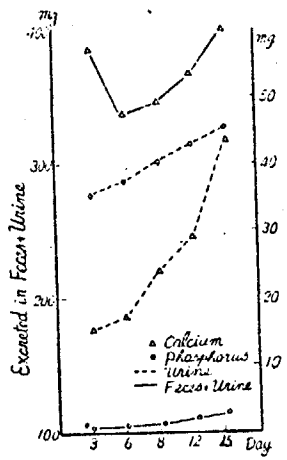
2. クエン酸ソーダ及びクエン酸カルシウムが家兎のカルシウムと燐の代謝に及ぼす影響

米糠と小麦麩を等量宛混合した飼料（以下Bran Dietと称する）で家兎を飼育すれば所謂骨軟症に罹患すると飯塚[9]は報告し，これが予防及び治療には炭酸カルシウムを飼料に添加することが必要であると報告している。

我々はこの飼料によつつて飼育された家兎のカルシウム及び燐の代謝が正常と如何に異つているかを始めに試験した。この結果は第2図の如くである。

燐，カルシウム共に排泄量が摂取量よりも多く，試験期間の末期になる程排泄量は増加している。これを尿中で観察すれば，試験日数の経過につれて，燐の排泄量は漸増するがカルシウムでは

Chart 2. Metabolism of Ca and P in Rabbits Reared with Bran Diet



Note: Assuming that the intake of Ca and P is 100mg each.

この結果とは異つて急激に上昇し試験末期には燐の排泄量と殆んど同量となつている。体内の調定塩基中カルシウムよりもナトリウム，加里が優先して排泄されることは飯塚[9]がアシドーシスの際に認められている。この試験でも始めにナトリウム，加里が排泄されて，試験日数の経過につれてカルシウムの排泄が増量されたので，穀類偏重飼料で家兎はアシドーシスとなり燐酸塩系の緩衝作用に随伴した現象であろう。

我々は1の実験からクエン酸ソーダがカルシウムの体内保持に効果のあることを認め，又クエン酸カルシウムは炭酸カルシウムよりもカルシウムの体内蓄積に効果あることを確めているから，この実験で得られたカルシウム排泄過剰の試験動物によつて，その効果を示す機転を解明しようとした。

1. ナトリウム塩類のカルシウム及燐代謝に及ぼす影響

クエン酸ソーダと炭酸ソーダを Bran Diet に10%の割合に添加した飼料で飼育した家兎のカルシウム及び燐代謝に及ぼす影響は第3表の如くである。クエン酸ソーダを添加した第1群は燐の排泄過剰とカルシウムの平衡を示し，クエン酸ソーダによつてカルシウムの排泄過剰を防止することが出来た。炭酸ソーダを添加した第2群では尿と糞の合計では燐もカルシウムも排泄過剰であつたが，尿中のカルシウム排泄量は著しく減少し

Reared with Bran Diet Supplemented with Sodium Citrate or Sodium Carbonate

Dietary Supplement	Number of Animals	Analysed Element	Intake (1)	Excreta (2)	Balance (1)-(2)	Proportion of Excreta to Intake: Intake	Excreta	Urine	Feces
Sodium Citrate	4	Calcium	49mg	49mg	0mg	100	100	33	67
		Phosphorus	732	1110	−378	100	152	70	82
Sodium Carbonate	4	Calcium	23	456	−433	100	198	10	183
		Phosphorus	344	474	−130	100	137	86	51

た。故に添加した化合物に共通しているナトリウム・イオンの影響でカルシウムの排泄が抑制されたことが分る。両群の間で糞中のカルシウム排泄量に差のあるのは，クエン酸ソーダ及び炭酸ソーダ共に溶解度の高い化合物であるが，腸内で遊離したカルシウムと前者はクエン酸カルシウム，後者は炭酸カルシウムを形成する可能性があつて，この生成物は前者は溶解度が高く後者は溶解度が低いためにこの差が生じたものと思われる。

Ⅱ．クエン酸のカルシウム及び燐代謝に及ぼす影響

この試験は家兎を Bran Diet で6日間飼育し後3日間の平均1日のカルシウム及び燐の摂取及び排泄量を測定し，その後6日間を Bran Diet にクエン酸10%を混じた飼料で飼育して同じく後3日間で代謝量を測定し比較したものである。

結果は第4表の如くとなり，Bran Diet にクエン酸を加えることによりいくらか燐の排泄量を抑制することが出来た。

これはクエン酸が体内では完全燃焼によつてアルカリとして作用するからいくらかアシドーシス防ぐ作用があるので，その結果燐の排泄量を減じたものと思われる。

Table 4. Metabolism of Calcium and Phosphorus in Rabbits Reared with Bran Diet Supplemented with Citric Acid

Dietary Supplement	Analysed Element	Intake (1)	Excreta (2)	Balance (1)-(2)	Proportion of Excreta to Intake: Intake	Excreta	Urine	Feces
None	Calcium	41mg	187mg	−146mg	100	456	10	90
	Phosphorus	632	744	−112	100	117	55	45
Citric Acid	Calcium	44	238	−194	100	543	2	93
	Phosphorus	673	750	− 77	100	111	43	57

Note : The same five male rabbits were employed in two kinds of the experiments.

Ⅲ．クエン酸カルシウム及び炭酸カルシウムのカルシウム及び燐代謝に及ぼす影響

以上の実験からカルシウムを与えるにもクエンカルシウムでは，その吸収及び蓄積に差異があると思われるので次の実験を行つた。

先ず主としてカルシウムの吸収を見るために試験始6乃至10日目に各4匹の家兎を殺し，食道を除いて消化管の内容物を水で洗い出し，それを灰化してカルシウムと燐とを定量し，同時にその日1日分の尿糞も集めて，カルシウムと燐との代謝量も測定した。飼料は Bran Diet 100g に第1群ではクエン酸カルシウム 7.5g，第2群では炭酸カルシウム 4g を添加し，両群共に飼料中の燐とカルシウムの比率を 1.5 に補正した飼料で飼育した。

試験の結果は第5表の如くで，両群共にカルシウムと燐は体内に蓄積された。しかし第1群の方が全体に蓄積され，消化管内に残つているカルシウムは第1群が第2群よりも少かつた。故に第1群の方がカルシウムの吸収，蓄積共に多かつた。

Table 5. Metabolism of Calcium and Phosphorus in Male Rabbits Reared with Bran Diet Supplemented with Calcium Citrate or Calcium Carbonate

Dietary Supplement	Number of Animals	Length of Period (Days)	Analysed Element	Intake (1)	Excreta (2)	Balance (1)-(2)	Residue in Intestine	Proportion of Excreta to Intake: Intake	Excreta	Urine	Feces
Calcium Citrate	4	6	Calcium	1114mg	379mg	+735mg	674mg	100	34	2	32
			Phosphorus	1703	1031	+627	1032	100	63	6	57
	4	10	Calcium	1194	786	+408	899	100	66	1	65
			Phosphorus	1830	1066	+764	1172	100	58	3	55
Calcium Carbonate	4	6	Calcium	1357	1233	+ 74	783	100	95	16	79
			Phosphorus	2018	1397	+621	1751	100	69	7	62
	4	10	Calcium	1357	1165	+192	1795	100	86	7	79
			Phosphorus	2018	1258	+760	2312	100	62	9	53

次に所謂骨軟症に罹患させた家兎に対する両化合物の影響を試験した。カルシウム欠乏飼料で長く飼育した家兎と正常飼育でカルシウムの体内蓄積の多い家兎では，たとえ炭酸カルシウムを与えた場合でもカルシウムの吸収される量は異なり，蓄積も前者が多くなるものである。故に家兎を60日間 Bran Diet で飼育して所謂骨軟症に罹患させた後に，前の試験と同様にクエン酸カルシウムと炭酸カルシウムを添加して夫々第1群と，第2群とし，6日間の予備試験を行い，後3日間の平均1日の摂取及び排泄したカルシウム及び燐量を測定してその結果を比較した。

この試験結果は第6表の如くとなつた。6日間の試験期間に両群共に体重は増加していた。第1群ではカルシウムも燐も蓄積を示し，第2群ではカルシウムは蓄積されるが燐は依然排泄過剰となつている。尿中のこれらの排泄量を見てもカルシウムでは第1群は第2群よりも少く，カルシウムを体内に蓄積させるのに炭酸カルシウムよりクエン酸カルシウムが勝つている。

Table 6. Metabolism of Calcium and Phosphorus in Male Rabbits Reared with Bran Diet Supplemented with Calcium Citrate or Calcium Carbonate after Reared with Bran Diet for 60 Days

Dietary Supplement	Number of Animals	Length of Period (Days)	Analysed Element	Intake (1)	Excreta (2)	Balance (1)-(2)	Proportion of Excreta to Intake: Intake	Excreta	Urine	Feces
Calcium Citrate	5	6	Calcium	1285mg	920mg	+365mg	100	72	6	66
			Phosphorus	1276	1243	+ 33	100	97	15	82
Calcium Carbonate	5	6	Calcium	919	853	+ 66	100	93	18	75
			Phosphorus	1303	1323	− 20	100	102	12	90

以上の試験結果から一般に広く用いられている炭酸カルシウムよりもクエン酸カルシウムがカルシウム剤としては勝り，特に穀類偏重飼料によつてアシドーシスが誘起されていると思われる場合に飼料に添加するカルシウム剤は有機酸のナトリウム塩類又はカルシウム塩類を使用することが必要であると思う。

結　　言

(1) 白鼠及び家兎を用い，クエン酸，クエン酸ソーダ及びクエン酸カルシウムのカルシウム及び燐代謝に及ぼす影響を試験した。

(2) ナトリウム・イオンはカルシウムの排泄を減少させ，穀類偏重飼料のカルシウム代謝に及ぼす影響を是正する。

(3) クエン酸カルシウムは炭酸カルシウムよりもカルシウムの吸収，蓄積を大にし，特に所謂骨軟症の予防，治療には良好なカルシウム剤である。

終に御校閲を頂いた当所生理科長長畑博士に深謝します。

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On the Influence of Calcium Citrate upon Calcium Metabolism

By T. NAKAJIMA and J. OTSUKA

As calcium is generally insufficient in daily feed for necessary demand of domestic animals in Japan, calcium carbonate is supplemented to the feed of almost all animals. From the results of the present experiment the authors intend to insist that calcium citrate is superior to calcium carbonate for the purpose.

They employed albino rats and rabbits in the experiment. The rats were fed unpolished rice supplemented with sodium citrate, calcium citrate or calcium carbonate for 75 days. After the observation of body growth during the feeding (Chart 1), the analyses of components of their bones were made (Table 2). Adult rabbits were fed equal mixture of rice- and wheat-bran supplemented with each of the three chemicals for about several days. Metabolism of calcium and phosphorus was estimated (Table 3, 4, 5, 6).

The results obtained were summarized as follows:

(1) When the animals were fed bran diet plus sodium citrate, the calcium retention of the animals increased to a considerable extent. It was considered from the results of additional experiments using sodium carbonate or citric acid that the good effect was mainly due to the action of Na-ions.

(2) When the animals were fed bran diet plus calcium citrate, the precipitated calcium in the bone was remarkably more than that of the animals with the bran plus calcium carbonate.

From: Klin, Wochenschrift, vol. 39, no. 20, p. 1064-1070.

Experimental investigations of intestinal calcium resportion as a function of the stomach acidity in man.

By Werner Niepmann.

From the II. Med.Clinic and Polyclinic of the Free University of Berlin (director: Priv.Doz.Dr.J.M. Schmitt-Rohde).

Introduction and problem.

Despite the central role which calcium resportion plays in the regulation of the calcium metabolism, the conditions of the resorption in the specific case are little known. The following factors are considered today as playing a role in intestinal calcium resportion under physiological conditions:

1. From the diet: fats (8,20.31,36,60,77); proteins (20,38,41,50,53,59,90); sugar (77,90); vitamin D (3,20,41); citric acid (20, 28,38,46,47,56,66,83) ascorbic acid (56,76); phytin (10,20,29,32,33,34,38,41,66,67,89); oxalic acid (9,14,20,38); phosphorus (20,38,41,67,77,85).
2. Body factors: bile (5,7,15,20,35,41,45,54,78,86); pH of gastric and intestinal juice (20,36,38,41,57,58,66,74).

The site of the calcium resportion is almost exclusively in the head part of the small intestine, particularly in the duodenum, where the stomach acid is not yet neutralized by the bases of the duodenal juice and where a clearly acid reaction with pH-values between 2.3 and 2.7 normally takes place. For this reason the major part of the calcium supplied with the food is in soluble and therefore resorbable form.

Though an influence of the stomach acid on the calcium resorption is generally assumed, there is hardly any experimental evidence available in man. Only Maurer and coworkers (57,58) could show with radioactive Ca (45) a direct relation between stomach acidity and calcium resorption. But in contrast to Hollinger and coworkers, 1956 (36), we think that the same calcium resorption is also possible with anacidity, and that the stomach acid is not essential for this process.

Our objective in this study- particularly in view of therapeutic consequences, is therefore to investigate the influence of the stomach acid on the intestinal calcium resorption in man after oral application of two different calcium compounds.

In this special problem the following facts have to be considered in the selection of a suitable investigation method.

Calcium balance tests, as far as they are carried out over longer periods of time (67), are suitable particularly for measuring the utilization and not the resorption, since an unresorbed and a resecreted calcium portion appears inthe feces, as known (6,13,39,48,67,75). Besides, considerable amounts of calcium are secreted uncontrolled through the sweat.

We excluded tests with radioactive Ca (45), because of the long half life period of Ca(45), 152 days). particularly since the respective authors recommend only a limited application of the method (49).

The result of animal tests with calcium resportion on the other hand must be applied very carefully to man, since - apart from the inner-secretory regulation - peculiarities exist between the individual species, particularly as far as calcium resorption is concerned (e.g. rat phytase (51), pH in duodenum in pigs with 3-6 more alkaline than in man(66) and others).

For comparative calcium resorption investigations, the determination of the increase of the calcium concentration in the serum after oral application of calcium compounds offers itself for theoretical considerations. But this method has already been used repeatedly to demonstrate calcium resorption in man (2,4,11,16-18,27,40,43,52,55,75,79,81,88). But these tests have always been carried out only on a few patients and show extremely great deviations from author to author, even from test to test, which go beyond the extent of a justifiable error margin.

Thus Siwe (79) observed in normal persons after the application of 0.74 g calcium as calcium chloride maximum concentration increases in the serum from 2-4 mg%, values which exceed partly even the results by Foscarini and coworkers (22) obtained after i.v. injection of the same dose. The same in-

crease was also observed by Schlossmann (81) with oral application of 7.4 g(!) calcium as calcium chloride. But Lasch and Neumayer (52) obtained an increase of 7.0 mg% under the same conditions.

By using calcium gluconate as a test substance, Liebermann (55) obtained after application of 0.9 g calcium maximum concentration increases of 11 mg%(!). Hein, on theother hand, obtained only 1.5 mg% after 0.72 g calcium (27).

These examples, which can be increased at random, led Klinke in 1954 to the finding that no characteristic increases in the serum calcium can be found in the normal person after oral application of calcium salts - regardless of whether they are soluble or difficultly insoluble, ionized or not ionized. None of the detectable fluctuations would withstand a statistical reexamination.

In perusing the literature of interest here, we find the surprising fact that none of the investigations shows a check of the results on a statistical basis. But since comparative investigations with great deviations of the values do not have any conclusive value in our opinion without such tests, we compared the results of our investigations and ascertained the differences statistically.

Procedure.

After oral application of calcium compounds, the calcium resorption in normal men and in patients with histamine-refractory anacidity was measured from the resorption curve in the serum, and the results were compared with each other. In all patients the acid values had been determined with fractioned siphoning. If in the histamine-refractory anacidics the probe findings dated some time back (up to ½ year), a control was made 2 days before the test with "desmoid". We confined ourselves to the comparison of normal acidics with histamine-refractory anacidics, since Schunk and Schmitt (84) could show great physioogical fluctuations of the acid values, particularly in the non-histamine-refractory anacidic range. Since the calcium-resorption depends among others on the need of the organism (23), the calcium supply was standardized 4 days before the test by giving the patients a low-

calcium diet (no milk and no cheese as the most important calcium carriers).

The tests were carried out on an empty stomach since no useful results are obtained if food is supplied at the same time (5,6).

We used an acid and a neutral calcium compound.

1. Neutral calcium carbonate.
2. Acid calcium citrate compound.

A commercial preparation was used which contained according to the manufacturer, in addition to 5% calcium and 30% citric acid, also 2.5% phosphate and 1% vitamin C (Dago C by Deutsche Arzneimittel-Gesellschaft, Berlin-Friedenau). (hereafter simply called "calcium citrate compound"). This preparation proved particularly suitable in preliminary tests, since it showed no side effects, like nausea, diarrhea and abdominal pains, even in the doses required here.

The best blood sampling times for the complete determination of the calcium-resorption curve proved to be in preliminary tests: on an empty stomach, 1½, 2½, 3½, 4½ and 6½ hours. Though the resorption curve had not returned completely to the fasting value after 6½ hours, the test was stopped at that time in consideration of the test persons.

The dosage was in all test persons uniformly 25 mg Ca/kg body weight. If we keep in mind that the daily calcium need under the various conditions is indicated with 0.8 - 4.0 g (36,38,51,85) we can consider the administered calcium quantities as physiological. The dose was given to the patents always immediately after the blood sampling on an empty stomach - suspended or dissolved in 300 ml tap water. The pH-values of the Dago C-solutions were between 3.5 and 4.0. The control persons (fasting fluctuations) received only 300 ml tap water. The calcium found naturally in tap water (about 30mg) can be disregarded.

The calcium determination in the serum was effected complexometrically according toa method which has an error margin of only 0.1 mg% and which is described by us elsewhere in detail (69). Besides, double determinations were made in all blood samplings.

53 persons were available for the tests.

1. 10 control persons where the fasting fluctuation of the calcium concentration in the serum was determined during the test period of 6½ hours.
2. 10 normacidics where calcium carbonate was used as a test substance.
3. 10 histamine-refractory anacidics where calcium carbonate was likewise used.
4. 10 normacidics who received the above mentioned calcium citrate compounds.
5. 13 histamine-refractory anacidics who were likewise examined after application of calcium citrate(as under 4).

Since the maximum concentration increase in the serum after oral application of calcium under otherwise equal conditions is subject to great fluctuations, as described above, we calculated for each test the area of the associated curve, expressed in mg% x hours, and used it as a relative measure for the calcium resorption.

The results and their differences resp. were compared statistically, calculating c as the difference measured in units of the mean value difference. If c is greater than 3, the difference is ascertained (I am indebted to Dr. med. et phil. K. Freudenbert for his valuable advice and for checking the statistical calculations).

Table 1-5. Calcium concentration in the serum in mg% on fasting and after application of 25 mg Ca/kg body weight in 53 persons (5 test groups) during a test period of 6½ hours.

FA - fractioned pumping out of stomach, N - normacidic, A- histamine-refractory anacidic, $F = \int_0^{6.5} Ca\text{-}t\,dt - 6.5\ Ca\text{-}o$ in mg% hours.

$\bar{F}$ = mean value of the areas F in the test group in mg% .h ; $\overline{Ca}\text{-}t - \overline{Ca}\text{-}o$ = average calcium concentration variation relative to the starting value in the test group in mg%.

Table 1. Ten control persons (fasting fluctuation).

Current no.	age in years	Sex	FA	fasting	Ca-t = calcium in mg% $1\frac{1}{2}$ h	$2\frac{1}{2}$h	$3\frac{1}{2}$ h	$4\frac{1}{2}$ h	$6\frac{1}{2}$ h	F
1	32	M	N	10.4	10.3	10.4	10.3	10.4	10.4	-0.22
2	34	M	N	10.4	10.5	10.5	10.9	10.4	10.2	+0.52
3	45	M	A	10.1	9.9	9.9	10.0	10.0	10.0	-0.80
4	26	M	N	10.5	10.4	10.7	10.7	10.6	10.5	+0.42
5	22	M	N	10.8	10.7	10.7	10.8	10.8	10.8	-0.22
6	31	F	A	9.6	9.6	9.5	9.5	9.5	9.4	-0.55
7	30	F	A	9.8	9.99	9.5	9.8	9.8	9.8	-0.17
8	28	F	A	10.2	10.3	10.3	9.7	10.1	10.3	-0.33
9	35	F	A	10.1	10.1	9.8	10.0	10.1	10.0	-0.50
10	24	F	N	11.0	11.1	10.8	10.8	10.8	10.9	-0.67
			$\overline{Ca}_t - \overline{Ca}_o$ =	0.0	0.0	-0.1	0.0	0.0	-0.1	$\overline{F}_1$ = -0.25

Table 2. Calcium carbonate in ten normacidic test persons.

Current no.	Age in years	Sex	FA	Fasting	Ca-t = calcium in mg% $1\frac{1}{2}$ hg	$2\frac{1}{2}$ h	$3\frac{1}{2}$ h	$4\frac{1}{2}$ h	$6\frac{1}{2}$h	F
1	21	M	N	10.0	10.6	11.0	10.8	10.4	10.2	+3.35
2	23	M	N	10.3	10.9	11.3	11.6	11.1	10.7	+4.65
3	28	M	N	10.9	11.9	12.3	12.0	11.5	11.2	+4.95
4	30	M	N	10.8	11.7	12.0	12.3	11.9	11.3	+5.97
5	35	M	N	10.0	10.4	10.9	10.7	10.4	10.3	+3.00
6	23	F	N	10.6	11.2	11.8	11.4	11.2	11.1	+4.15
[illegible]	25	F	N	9.3	10.0	10.4	10.4	10.1	9.7	+4.67
8	26	F	N	9.6	10.3	10.7	11.2	10.8	10.1	+5.77
9	26	F	N	10.5	11.0	11.4	11.7	11.3	10.7	+4.12
10	32	F	N	10.2	10.6	10.9	11.4	10.9	10.8	+4.05
			$\overline{Ca}_t - \overline{Ca}_o$ =	0.0	+0.6	+1.1	+1.1	+0.7	+0.4	$\overline{F}_2$ = +4.47

Table 3. Calcium carbonate in 10 histamine-refractory anacidic patients.

Current	Age in	Sex	FA	Fasting	Ca-t = calcium in mg% $1\frac{1}{2}$ h	$2\frac{1}{2}$h	$3\frac{1}{2}$h	$4\frac{1}{2}$h	$6\frac{1}{2}$h	F
1	36	M	A	10.4	10.6	10.7	10.5	10.5	10.5	+0.90
2	40	M	A	9.1	9.2	9.3	9.1	9.1	9.1	+0.32
3	47	M	A	10.1	10.3	10.3	10.2	10.1	10.1	+0.55
4	48	M	A	9.6	9.9	10.0	9.8	9.7	9.6	+1.12
5	62	M	A	9.9	10.3	10.1	10.0	9.9	9.9	+0.80
6	25	F	A	9.6	10.0	9.9	9.9	9.8	9.7	+1.50
7	50	F	A	9.5	9.7	9.9	9.7	9.6	9.6	+1.10
8	51	F	A	10.0	10.3	10.2	10.2	10.1	10.1	+1.02
9	59	F	A	9.4	9.5	9.6	9.6	9.5	9.4	+0.57
10	60	F	A	10.0	10.3	10.3	10.0	10.0	10.1	+0.77
			$\overline{Ca}_t - \overline{Ca}_o$ =	0.0	+0.3	+0.3	+0.1	+0.1	0.0	$\overline{F}_3$ = +0.87

Table 4. Calcium citrate compound in ten normacidic test persons

Current no.	Age in years	Sex	FA	Fasting	Ca-t = calcium in mg% 1½h	2½ h	3½ h	4½ h	6½ h	F
1	21	M	N	10.2	10.9	11.0	11.0	10.7	10.3	+3.32
2	22	M	N	10.7	11.4	11.8	11.5	11.5	11.1	+4.32
3	23	M	N	10.9	11.8	12.2	12.3	11.7	11.3	+5.42
4	24	M	N	11.2	11.8	12.4	12.2	11.7	11.2	+3.70
5	24	M	N	9.6	10.3	10.5	10.4	10.6	10.1	+4.57
6	25	M	N	10.6	11.2	11.8	12.2	11.9	11.5	+6.40
7	27	M	N	10.5	11.1	11.7	11.4	11.0	11.1	+4.30
8	46	M	N	10.5	10.9	11.3	11.8	11.6	11.0	+4.75
9	30	F	N	9.3	9.6	10.0	10.5	10.4	9.7	+4.32
10	30	F	N	10.0	10.7	11.2	11.4	11.6	10.7	+6.57
				$\overline{Ca}_t - \overline{Ca}_o = 0.0$	+0.6	+1.0	+1.1	+0.9	+0.5	$\overline{F}_4 = +4.77$

Table 5. Calcium citrate compound in 13 histamine-refractory anacidics.

Current no.	Age in years	Sex	FA	Fasting	Ca-t = calcium in mg% 1½ h	2½ h	3½ h	4½ h	6½ h	F
1	47	M	A	9.1	9.8	10.0	9.9	9.9	9.4	+4.17
2	68	M	A	8.9	9.9	10.3	9.9	9.6	9.3	+5.85
	30	F	A	9.9	10.6	10.9	10.7	10.5	10.0	+3.67
4	34	F	A	10.0	10.6	10.8	10.6	10.5	10.3	+3.20
5	55	F	A	10.8	11.3	11.6	11.6	11.1	10.8	+2.67
6	57	F	A	10.2	10.8	11.3	11.1	10.7	10.2	+3.50
7	62	F	A	9.6	10.2	10.5	10.6	10.3	10.0	+4.10
8	64	F	A	9.5	9.9	10.7	10.5	10.1	9.9	+4.00
9	51	M	A	10.0	10.1	10.3	10.2	10.0	10.0	+0.62
10	68	M	A	9.9	10.3	10.3	10.2	10.1	9.9	+1.50
11	57	F	A	10.0	10.1	10.2	10.0	9.9	9.7	-0.10
12	57	F	A	10.2	10.4	10.5	10.4	10.3	10.2	+0.87
13	65	F	A	10.1	10.5	10.6	10.3	10.4	10.1	+1.65
				$\overline{Ca}_t - \overline{Ca}_o = 0.0$	+0.5	+0.8	+0.6	+0.4	+0.1	$\overline{F}_5 = +2.75$

Results

The results of our tests are compiled in table 1 to 5 and in fig. 1-3. In the calculation of the areas F as a resorption measure, the actual course of the curves had to be replaced for obvious reasons by an approximate curve, since a linear course of the function Ca-t was assumed between 2 measuring points each and the respective area sections represent this way trapeazoids. The starting values were deducted from the average values of the calcium concentration at the time to so that $\overline{Ca}$-t - $\overline{Ca}$-o represents the average variations relative to the starting value.

The calculation of the mean deviation of the mean value of the areas $\overline{F}$ was effected as usual according to

$$s\overline{F} = \sqrt{\frac{SIGMA(F - \overline{F})^2}{n(n - 1)}}$$

For the individual test groups we obtained thus the following values.

1. 10 control persons (fasting fluctuation) $\overline{F}1 = -0.25 \pm 0.14$ mg% . h.
2. 10 healthy normacidics, pretested with calcium carbonate $\overline{F}2 = +4.47 \pm 0.30$ mg% . h.
3. 10 histamine-refractory anacidics likewise with calcium carbonate $\overline{F}3 = +0.87 \pm 0.11$ mg% . h.
4. 10 healthy normacidics who received the above mentioned calcium citrate compound $\overline{F}4 = +4.77 \pm 0.34$ mg% . h.
5. 13 histamine-refractive anacidics likewise with calcium citrate $F5 = +2.75 \pm 0.48$ mg% . h.
6. In 25 normacidics the fasting calcium concentration in the serum (starting values) was $\overline{Ca}\text{-}ON = 10.4 \pm 0.10$ mg. h.
7. in 28 histamine-refractory patients the fasting value (starting value) was $\overline{Ca}\text{-}OA = 9.5 \pm 0.10$ mg%

The test of the difference was made, as mentioned above, by calculating

$$c = D/sD$$

where D is the difference of the mean values to be compared . sD represents the mean deviation of the mean value difference

$$sD = \sqrt{s^2_{\overline{x}1} + s^2_{\overline{x}2}} ,$$

where $\overline{x}$ denotes an arithmetic mean in general.

Fig. 1. Average calcium fasting fluctuation in the serum in 10 control persons (according to tsble 1).

Fig. 2. 25 mg Ca/kg as calcium chloride given orally. Average calcium concentration increases in the serum in 10 normacidics _____ (accord. to table 2) and in 10 histr. refr. anacidics -.-.-.- (accord. to table 3).

Fig. 3. 25 mg Ca/kg as calcium citrate compounds given orally. Average calcium-concentration increase in the serum in 10 noracids ___(according to table 4) and in 13 hist. refr. anacidics -.-.-.- (according to table 5).

We obrain thus in the following comparisons:

Mean values	D	c	mean values	D	c
$\overline{F}2 - \overline{F}1$	+4.72	14.0	$\overline{F}2 - \overline{F}3$	+3.60	11.2
$\overline{F}3 - \overline{F}1$	+1.12	6.6	$\overline{F}4 - \overline{F}5$	+2.02	3.4
$\overline{F}4 - \overline{F}1$	+5.02	13.9	$\overline{F}5 - \overline{F}3$	+1.88	3.9
$\overline{F}5-\overline{F}1$	+3.00	6.0	$\overline{Ca}_{ON}-\overline{Ca}_{OA}$	+0.9	6.4
$\overline{F}4 - \overline{F}2$	+0.30	0.7			

Discussion

In order to be able to provide information about the calcium concentration increase in the serum after oral application of calcium, we had to determine first the size of the physiological fluduation of the calcium content in the human serum during the test period. Unfortunately few data are available in the literature on this subject.

Thus Vollmner (88) found maximum deviations of 0.5 mg% during a 6 hour test period in 4 tests. Siwe (79) reports in 8 3-hour tests about maximum variations of 1.0 mg% and Kircher (44) obtained in 34 children as an average difference between 2 blood samplings within 1½ - 2 hours 0.55 mg% calcium. In 7 children the difference was greater, however, than 1 mg%.

Since these data were not sufficient for our purpose, we made our own tests. As it can be seen from table 1, maximum concentration fluctuations between 0.1 and 0.7 mg% were observed in 10 control persons. The average variations relative to the starting value (Ca-t - Ca-o) however, differ from each other by not more than 0.1 mg% in the various sampling times so that a sufficient basis for the following statistical comparisons was given. The mean value of the areas is -0.25 ± 0.14 mg%,h, and its deviation from 0 is even within the random deviation.

As mentioned above, the error margin of the complexometric calcium deter-

mination method in the serum used is only ±0.1 mg% (69). Besides, double determinations were always made. Since possible measuring errors are reduced by the use of the mean values of the concentration variations in 10 and 13 test persons resp., methodic error possibilities can be disregarded in our considerations. When using the mean values of the area as a measure for the calcium resorption of the respective test group, the following resorption curves obtained after oral application of 25 mg Ca/kg body weight can be delimited from the fasting fluctuation as not accidental (c greater than 3):

1. Calcium carbonate in healthy normacidics (table and fig. 2).
2. Calcium carbonate in histamine-refractory anacidics (table 3 and fig. 2).
3. Calcium citrate compound in healthy noramcidics (table 4 and fig. 3).
4. Calcium citrate compound in histamine-refractory anacidics (table 5 and fig. 3).

It has thus been proven that the resorption of orally applied calcium compounds can be detected and checked resp. by determining the calcium content in the serum. The dosage remained in the physiological range (1.75 g calcium for a person weighing 70 kg, for example).

Beyond the strictly qualitative resorption test it is possible with the method to make comparative statements in the various test groups. Though the resorption of the calcium citrate compound with 4.77 mg%.h in the normacidic is slightly greater than that of calcium carbonte (4.47), this difference can not be ascertained statistically (c = 0.7). In healthy persons with normal stomach acidity we can therefore find no reliable difference between the resorption of these two calcium compounds.

But the resorption of the two compounds is significantly lower in patients without stomach acidity than in the normacidics because there is a differene insofar as the acid calcium citrate compound applied is clearly better resorbed in the histamine-refractory anacidics than the neutral calcium carbonate (D = 1.88; c = 3.9).

But in the 5th test group we notice the relatively great mean deviation with almost ± 0.5 mg%.h. In fact the patients 1-8 of this group show a mean calc-

ium-resorption value of +3.90 ± 0.33 mg%.h, the test persons 9 to 13, however, only a value of +0.91 ± 0.32 mg%.h. Here we could think perhaps of an additional factor interfering with the calcium resorption, for example, accelerated passage through the intestine or inflammatory or degenerative processes of the intestinal mucuous membrane etc.

Table 2-5 and fig. 2 and 3 show that the calcium resorption in the healthy normacidics was not yet completed after 6½ hours, while the anacidic curves had already attained again the starting value at that time.

The significance of the diseases of the stomach and intestine for the resorption of calcium has been pointed out repeatedly. Thus Holt and coworkers (37) and later Hallman and coworkers (26) among others observed a reduced calcium resorption in chronic digestive disorders. Recently Nicolaysen and Ragard (68) had found a highly negative calcium balance in 15 gastrectomized patents.

But clinically detectable calcium deficiences appear as a rule only if several factors (chronic calcium resorption disturbance, calcium deficiency diet or increased calcium consumption) appear in the same individual.

Naturally it is logical to bring an inadequate calcium supply and resorption in relation to other disturbances of the calcium metabolism in the sense of "alimentary osteophathies. (1,12,15,30,54,61-65,70,71,78,86,87). Particularly the significance of anacidity in the genesis of resorption-related calcipenic osteophathies in the sense of Barthelheimer and Schmitt-Rohde (5) has been pointed out repeatedly.

In 1938 Meulenbracht (64) already coined the term "osteomalacia achylica" for this phenomenon. His investigations were supplemented later a.o. by Pahlke (72) and only recently Franz (24) has dedicated a detailed study to the relations between lack of hydrochloric acid in the gastric juice and the genesis of calcipenic osteopathies.

With the proof of the disturbed resorption, particularly of neutral calcium-compounds with insufficient hydrochloric acid in the stomach, established with this study, the pathogenesis of these symptoms can be supplemented as

follows:

HCl deficiency of the stomach -- reduced calcium resorption - resorption-related calcipenic osteopathies.

If we consider that the decrease of the hydrochloric acid in the stomach is a physiological phenomenon in old age (for a recent summary review see Ritter (73)), we find from the resulting inadequate calcium resorption at least an important ethiological factor for the genesis of osteophathies in old age.

The small, though significant difference of the mean initial calcium concentration in the serum (fasting value) between the normacidic (10.4 mg%) and the anacidic test group (9.5 mg%) gains importance if we consider the higher average age in the second group (48.6 years against 27.2 years in the normacidics). According to tests by Elkinton and Danowski (19) on about 170 healthy persons of all age groups, an increase of the calcium concentration can rather be expected with increasing age. Though the serum calcium content must be kept constant by the depositary function of the bone tissue (Apitzsch (4) Jesserer (41), Schmitt-Rohde (82), there is a significant reduction of the serum calcium in chronic calcium resorption disturbances and the resulting chronic calcium deficiency.

Summary.

The intestinal calcium resorption was tested in 53 test persons after oral application of 25 mg calcium per kg body weight by determining the calcium content in the serum. To this end the following test groups were formed:

1. 10 control persons where the fasting fluctuation of the calcium concentration in the serum was determined during the test period.

2. 10 healthy persons with normal stomach acidity where calcium carbonate served as a test substance.

3. 10 histamine-refractory anacidic patients where calcium carbonate was likewise used.

4. 10 healthy normacidics who received a commercial calcium citrate compound (Dago C).

5. 13 histamine-refractory anacidics where the resorption was likewise tested with this calcium citrate compound.

As a measure for the size of the resorption we used the rarea of the respective curve, expressed in mg%.h. The tests were made on fasting patients. The test period was in all cases 6' hours.

The calcium in the serum was determined complexometrically. The results were compared statistically.

Our results show:

1. The results of orally administered calcium compounds can be detected and checked resp. by determining the calcium content in the serum.
2. Of the two calcium compounds used, the calcium citrate compound showed in healthy persons with normal stomach acidity a better resorption value, but the difference with regard to calcium carbonate could not be ascertained statistically.
3. The resorption of the two compounds in patients without stomach acidity however, is significantly lower, the acid calcium citrate compound being resorbed bettery by these people than the neutral calcium carbonate.
4. The fasting calcium concentration in the serum is significantly lower in the anacidic group with 9.5 mg% than the calcium content in the normacidics (10.4 mg%).

Reference

Translated by Carl Demrick Associates, Inc./IE/db

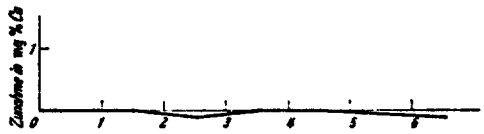

Figure 1

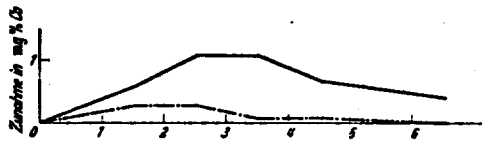

Figure 2

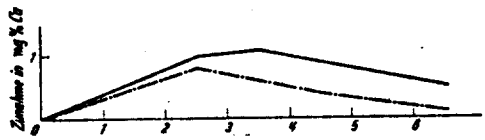

Figure 3

Serumeiweiße, d. h. also alle Substanzgruppen vermehrt, die als Rohstoffe für die Bildung des Blutfarbstoffes in Betracht kommen. Dagegen ist bei hypochromen Anämien nur immer der Teil jener Rohstoffe im Überschuß vorhanden, der wegen Fehlens eines anderen oder Ausfall eines Ferments, nicht für die Blutbildung verwertet werden kann. Unter diesem Blickwinkel wurde die vermehrte Porphyrinausscheidung auch bei der Tarda verschiedentlich auf eine Störung des Eiseneinbaues oder aber auf eine Störung der Protoporphyrinbildung durch einen Fermentblock zurückgeführt. Durch unsere Untersuchungen glauben wir gezeigt zu haben, daß bei dieser Porphyrie die Rohstoffe Eisen, Pyrrolderivate und Eiweiß reichlich vorhanden sind und normal verwertet werden, so daß die bei unseren Patienten beobachtete Hyperplasie des erythropoetischen Systems nicht Ausdruck irgendeines Mangels, sondern Folge eines reichlichen Rohstoffangebotes ist. Dank dieser günstigen Verhältnisse ist bei der Tarda der Entzug größerer Blutmengen mit günstigem therapeutischem Effekt möglich, wobei das hämatologische Verhalten des Spätporphyrikers unter solchen Aderlässen schließlich als weitere Stütze für die vorstehend entwickelten Anschauungen gewertet werden kann.

Zusammenfassung. 24 Fälle von Porphyria cutanea tarda wurden hämatologisch näher untersucht.

Während das periphere Blut außer dem meist erhöhten Serumeisen-Spiegel normale Werte zeigte, fand sich im Knochenmark häufig eine erythroblastische Hyperplasie mit Hämosiderose, jedoch ohne Sideroblastose. Zusammen mit den Beobachtungen an Tarda-Patienten unter therapeutischen Aderlässen, der Bestimmung der Erythrocytenlebensdauer und der Untersuchung des Pyridoxin-Stoffwechsels deuten die Befunde bei der Spätporphyrie u. E. auch hämatologisch auf eine enge Verwandtschaft mit der Hämochromatose, während nähere Beziehungen zu den hämolytischen und sideroachrestischen Anämien weitgehend ausgeschlossen werden konnten. Ebenso sprechen die Untersuchungen gegen eine wesentliche Bedeutung von Eiseneinbaustörungen oder Fermentblockierungen in der Pathogenese dieser Porphyrieform.

Die Deutsche Forschungsgemeinschaft unterstützte die Untersuchungen in dankenswerter Weise.

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Experimentelle Untersuchungen zur intestinalen Calcium-Resorption in Abhängigkeit von der Magenacidität beim Menschen

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Einleitung und Problemstellung

Trotz der zentralen Rolle, die der Calcium-Resorption in der Regelung des Calcium-Stoffwechsels zuerkannt wird, überblicken wir im einzelnen die Bedingungen der Resorption nur unvollständig. Folgenden Faktoren mißt man heute bei der intestinalen Calcium-Resorption unter physiologischen Bedingungen Bedeutung bei:

1. Aus der Nahrung: Fette[8, 20, 31, 36, 60, 77]; Proteine[20, 36, 38, 41, 50, 53, 59, 90]; Zucker[36, 77, 90]; Vitamin D[3, 20, 41]; Citronensäure[20, 28, 38, 46, 47, 56, 66, 83]; Ascorbinsäure[56, 76]; Phytin[10, 20, 29, 32, 33, 34, 38, 41, 66, 67, 89]; Oxalsäure[9, 14, 20, 38]; Phosphor[20, 38, 41, 67, 77, 85].

2. Körpereigene Faktoren: Galle[5, 7, 15, 20, 35, 41, 45, 54, 73, 86]; p_H des Magen- und Darmsaftes[20, 36, 38, 41, 57, 58, 66, 74].

Der Ort der Calcium-Resorption liegt nahezu ausschließlich im Anfangsteil des Dünndarms, vor allem im Duodenum, wo die Magensäure noch nicht von den Basen des Duodenalsaftes neutralisiert ist und wo normalerweise noch eine deutlich saure Reaktion mit p_H-Werten zwischen 2,3 und 2,7 herrscht. Deshalb befindet sich hier auch der größte Teil des mit der Nahrung zugeführten Calciums in löslicher und daher resorbierbarer Form.

Wenn auch im allgemeinen ein Einfluß der Magensäure auf die Calcium-Resorption angenommen wird, so liegen hierfür doch kaum experimentelle Beweise beim Menschen vor. Lediglich MAURER u. Mitarb.[57, 58] konnten mit radioaktivem Ca^{45} eine direkte Beziehung zwischen Magenacidität und Calcium-Resorption zeigen. Jedoch halten hierzu im Widerspruch HOLLINGER u. Mitarb. 1956[36] die gleiche Calcium-Resorption auch bei der Anacidität für möglich und betrachten die Magensäure für diesen Vorgang als entbehrlich.

Deshalb haben wir uns in dieser Arbeit — besonders im Hinblick auf therapeutische Konsequenzen — die Aufgabe gestellt, den Einfluß der Magensäure auf die intestinale Calcium-Resorption beim Menschen nach oraler Gabe von zwei verschiedenen Calcium-Verbindungen zu untersuchen.

Bei dieser speziellen Fragestellung ist zur Auswahl der geeigneten Untersuchungsmethode folgendes zu beachten:

MEDICAL

Calcium-*Bilanzuntersuchungen*, sofern sie über längere Zeit durchgeführt werden[47], sind vor allem zur Messung der Ausnutzung und nicht der Resorption geeignet, da im Stuhl bekanntlich eine nichtresorbierte und eine wieder ausgeschiedene Calcium-Fraktion erscheint[6, 13, 30, 48, 67, 75]. Außerdem werden nicht geringe Mengen an Calcium unkontrolliert durch den Schweiß ausgeschieden[42, 51, 89].

Auch Versuche mit *radioaktivem* Ca^{45} haben wir wegen der langen Halbwertzeit des Ca^{45} (152 Tage) abgelehnt, zumal die betreffenden Autoren teilweise selbst nur eine begrenzte Anwendung ihrer Methode empfehlen[19].

Die Ergebnisse von *Tierversuchen* wiederum sind im Calcium-Stoffwechsel nur mit Vorsicht auf den Menschen übertragbar, da — abgesehen von der innersekretorischen Regulation — auch gerade im Hinblick auf die Calcium-Resorption Besonderheiten bei den einzelnen Tierarten bestehen (z. B. Rattenphytase[51], p_H im Duodenum bei Schweinen mit 3—6 alkalischer als beim Menschen[66] u. a.).

Dagegen bietet sich für vergleichende Calcium-Resorptionsuntersuchungen aus theoretischen Erwägungen die Bestimmung des Calcium-Konzentrationsanstieges im Serum nach oraler Gabe von Calcium-Verbindungen an. Dieser Weg ist daher auch schon verschiedentlich zum Nachweis der Calcium-Resorption beim Menschen beschritten worden[2, 4, 11, 16–18, 27, 40, 43, 52, 55, 75, 79, 81, 88]. Allerdings sind diese Untersuchungen immer nur an wenigen Patienten durchgeführt worden und zeigen von Autor zu Autor, ja von Versuch zu Versuch, außerordentlich starke, oft über das Maß einer vertretbaren Fehlergrenze hinausgehende Streuungen:

So beobachtete SIWE[79] bei normalen Personen nach Gabe von 0,74 g Calcium als Calciumchlorid maximale Konzentrationsanstiege im Serum von 2—4 mg-% Calcium, Werte, die zum Teil sogar noch die nach i. v. Injektion der gleichen Dosis erhaltenen Ergebnisse von FOSCARINI u. Mitarb.[22] übersteigen! Den gleichen Anstieg beobachtete auch SCHLOSSMANN[81] bei oraler Verabreichung von 7,4 g (!) Calcium als Calciumchlorid. Dagegen erhielten LASCH und NEUMAYER[52] unter sonst gleichen Bedingungen eine Erhöhung von 7,0 mg-%.

Bei Verwendung von Calciumgluconat als Testsubstanz erhielt LIEBERMANN[55] nach Gabe von 0,9 g Calcium maximale Konzentrationsanstiege um 11 mg-% (!), HEIN dagegen nach 0,72 g Calcium nur etwa 1,5 mg-%[27].

Diese Beispiele, die sich noch beliebig vermehren lassen, veranlaßten KLINKE noch 1954[47] zu der Feststellung, daß beim normalen Menschen unter der oralen Gabe von Kalksalzen — ganz gleich ob löslich, schwer löslich, ionisiert oder nicht ionisiert — keinerlei kennzeichnende Erhöhungen im Serum-Calcium nachgewiesen werden können. Sämtliche feststellbaren Schwankungen würden einer statistischen Nachprüfung nicht standhalten.

Es ergibt sich nämlich bei der Durchsicht der hier interessierenden Literatur die überraschende Tatsache, daß die Untersuchungen ausnahmslos eine Prüfung der Ergebnisse auf statistischer Grundlage vermissen lassen. Da unseres Erachtens aber vergleichende Untersuchungen mit starker Streuung der Werte ohne eine derartige Prüfung wenig Aussagekraft besitzen, haben wir die Ergebnisse unserer Untersuchungen verglichen und die Differenzen statistisch gesichert.

Methodik

Nach oraler Gabe von Calcium-Verbindungen wurde beim Menschen mit normalen Magensäurewerten und bei Patienten mit histaminrefraktärer Anacidität die Calcium-Resorption aus der Resorptionskurve im Serum gemessen und miteinander verglichen. Bei allen Patienten waren die Säurewerte mit der fraktionierten Ausheberung ermittelt worden. Wenn bei den histaminrefraktär Anaciden der Sondenbefund schon längere Zeit (bis zu $^1/_2$ Jahr) zurücklag, wurde 2 Tage vor dem Versuch eine Kontrolle mit „Desmoid" durchgeführt. Wir beschränkten uns auf den Vergleich Normacider zu histaminrefraktär Anaciden, da SCHUNK und SCHMITT[64] besonders im nichthistaminrefraktär anaciden Bereich starke physiologische Schwankungen der Säurewerte zeigen konnten. Da die Calcium-Resorption u. a. vom Bedarf des Organismus abhängt[23], wurde die Calciumzufuhr 4 Tage vor Versuch standardisiert, indem die Patienten eine calciumarme Kost (keine Milch und kein Käse als wichtigste Kalkträger) erhielten.

Die Versuche wurden an nüchternen Personen durchgeführt, da bei gleichzeitiger Nahrungszufuhr keine brauchbaren Ergebnisse erzielt werden[5, 6].

Zur Anwendung gelangten eine neutrale und eine saure Calcium-Verbindung:

1. Neutrales Calciumcarbonat.
2. Saure Calciumcitrat-Verbindung. Hierbei bedienten wir uns eines handelsüblichen Präparates, das nach Angaben der Herstellerfirma neben 5% Calcium und 30% Citronensäure noch 2,5% Phosphat und 1% Vitamin C enthält* (im folgenden einfach als „Calciumcitrat-Verbindung" bezeichnet). Dieses Präparat erwies sich in Vorversuchen besonders geeignet, da es auch in der hier notwendigen Dosierung in keinem Fall Nebenwirkungen wie Übelkeit, Durchfälle und Leibschmerzen zeigte.

Als günstigste Blutentnahmezeiten zur vollständigen Erfassung der Calcium-Resorptionskurve erwiesen sich in Vorversuchen: nüchtern, $1^1/_2$, $2^1/_2$, $3^1/_2$, $4^1/_2$ und $6^1/_2$ Std. Obwohl nach $6^1/_2$ Std die Resorptionskurve oft noch nicht ganz zum Nüchternwert zurückgekehrt war, wurde zu diesem Zeitpunkt der Versuch mit Rücksicht auf die Versuchspersonen abgebrochen.

Die Dosierung betrug bei allen Versuchspersonen gleichmäßig 25 mg Ca/kg Körpergewicht. Wenn man bedenkt, daß der tägliche Calciumbedarf unter den verschiedenen Bedingungen mit 0,8—4,0 g angegeben wird[36, 38, 51, 85], so dürfen wir die von uns verabreichten Calcium-Mengen als physiologisch bezeichnen. Die Dosis wurde den Patienten jeweils unmittelbar nach der Nüchternentnahme (Ausgangswert) verabfolgt — aufgeschwemmt bzw. gelöst in 300 ml Leitungswasser. Die p_H-Werte der Dago C-Lösungen lagen zwischen 3,5 und 4,0. Die Kontrollpersonen (Nüchternschwankung) erhielten jeweils nur 300 ml Leitungswasser. Das in 300 ml Wasser natürlich vorkommende Calcium (etwa 30 mg) kann vernachlässigt werden.

Die Calcium-Bestimmung im Serum erfolgte komplexometrisch nach einer Methode, die eine Fehlerbreite von nur 0,1 mg-% aufweist und die an anderer Stelle von uns eingehend beschrieben worden ist[69]. Außerdem wurden bei allen Blutentnahmen Doppelbestimmungen durchgeführt.

Für die Versuche standen 53 Personen zur Verfügung:

1. 10 Kontrollpersonen, bei denen die Nüchternschwankung der Calcium-Konzentration im Serum während der Versuchsdauer von $6^1/_2$ Std ermittelt wurde.
2. 10 Normacide, bei denen Calciumcarbonat als Testsubstanz diente.
3. 10 histaminrefraktär anacide Patienten, bei denen ebenfalls Calciumcarbonat verwendet wurde.
4. 10 Normacide, die die oben erwähnte Calciumcitrat-Verbindung erhielten.
5. 13 histaminrefraktär Anacide, die ebenfalls nach Gabe von Calciumcitrat (wie unter 4) untersucht wurden.

Da — wie oben beschrieben — der maximale Konzentrationsanstieg im Serum nach oraler Kalkgabe unter sonst gleichen Versuchsbedingungen starken Schwankungen unterliegt, wurde von uns für jeden Versuch die Fläche der zugehörigen Kurve, ausgedrückt in mg-% × Std berechnet und dieses als relatives Maß für die Calcium-Resorption benutzt.

Die Ergebnisse bzw. deren Differenzen wurden statistisch verglichen, wobei c als die in Einheiten der mittleren Abweichung der Mittelwertdifferenz gemessene Differenz der zu vergleichenden Mittelwerte berechnet wurde. Ist c größer als 3, dann ist die Differenz gesichert**.

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* „Dago C" der Deutschen Arzneimittel-Gesellschaft, Berlin-Friedenau.

** Herrn Professor Dr. med. et phil. K. FREUDENBERG danke ich für seine wertvollen Hinweise und die Überprüfung der statistischen Berechnungen.

Tabelle 1—5. *Calciumkonzentration im Serum in mg-% nüchtern und nach Gabe von 25 mg Ca/kg Körpergewicht bei 53 Personen (5 Versuchsgruppen) während einer 6½stündigen Versuchsdauer*

Die Zahlen stellen Mittelwerte von Doppeltitrationen dar.

FA = fraktionierte Magenausheberung; N = normacid; A = histaminrefraktär anacid; $F = \int_0^{6,5} Ca_t\,dt - 6,5\,Ca_0$ in mg-% · Std; $\bar{F}$ = Mittelwert der Flächen F bei der Versuchsgruppe in mg-% · Std; $\overline{Ca_t} - \overline{Ca_0}$ = durchschnittliche Calciumkonzentrationsveränderung gegenüber dem Ausgangswert bei der Versuchsgruppe in mg-%.

Tabelle 1. *Zehn Kontrollpersonen* (Nüchternschwankung)

Lfd. Nr.	Alter in Jahren	Geschlecht	FA	Ca_t = Calcium in mg-%						
				Nüchtern	1½ Std	2½ Std	3½ Std	4½ Std	6½ Std	F
1	32	♂	N	10,4	10,3	10,4	10,3	10,4	10,4	−0,22
2	34	♂	N	10,4	10,5	10,5	10,9	10,4	10,2	+0,52
3	45	♂	A	10,1	9,9	9,9	10,0	10,0	10,0	−0,80
4	26	♂	N	10,5	10,4	10,7	10,7	10,6	10,5	+0,42
5	22	♂	N	10,8	10,7	10,7	10,8	10,8	10,8	−0,22
6	31	♀	A	9,6	9,6	9,5	9,5	9,5	9,4	−0,55
7	30	♀	A	9,8	9,9	9,5	9,8	9,8	9,8	−0,17
8	28	♀	A	10,2	10,3	10,3	9,7	10,1	10,3	−0,33
9	35	♀	A	10,1	10,1	9,8	10,0	10,1	10,0	−0,50
10	24	♀	N	11,0	11,1	10,8	10,8	10,8	10,9	−0,67
			$\overline{Ca_t} - \overline{Ca_0}$ =	*0,0*	*0,0*	*−0,1*	*0,0*	*0,0*	*−0,1*	$\bar{F}_1 = -0,25$

Tabelle 2. *Calciumcarbonat bei zehn normaciden Versuchspersonen*

Lfd. Nr.	Alter in Jahren	Geschlecht	FA	Ca_t = Calcium in mg-%						
				Nüchtern	1½ Std	2½ Std	3½ Std	4½ Std	6½ Std	F
1	21	♂	N	10,0	10,6	11,0	10,8	10,4	10,2	+3,35
2	23	♂	N	10,3	10,9	11,3	11,6	11,1	10,7	+4,65
3	28	♂	N	10,9	11,9	12,3	12,0	11,5	11,2	+4,95
4	30	♂	N	10,8	11,7	12,0	12,3	11,9	11,3	+5,97
5	35	♂	N	10,0	10,4	10,9	10,7	10,4	10,3	+3,00
6	23	♀	N	10,6	11,2	11,8	11,4	11,2	11,1	+4,15
7	25	♀	N	9,3	10,0	10,4	10,4	10,1	9,7	+4,67
8	26	♀	N	9,6	10,3	10,7	11,2	10,8	10,1	+5,77
9	26	♀	N	10,5	11,0	11,4	11,7	11,3	10,7	+4,12
10	32	♀	N	10,2	10,6	10,9	11,4	10,9	10,8	+4,05
			$\overline{Ca_t} - \overline{Ca_0}$ =	*0,0*	*+0,6*	*+1,1*	*+1,1*	*+0,7*	*+0,4*	$\bar{F}_2 = +4,47$

Tabelle 3. *Calciumcarbonat bei zehn histaminrefraktär anaciden Patienten*

Lfd. Nr.	Alter in Jahren	Geschlecht	FA	Ca_t = Calcium in mg-%						
				Nüchtern	1½ Std	2½ Std	3½ Std	4½ Std	6½ Std	F
1	36	♂	A	10,4	10,6	10,7	10,5	10,5	10,5	+0,90
2	40	♂	A	9,1	9,2	9,3	9,1	9,1	9,1	+0,32
3	47	♂	A	10,1	10,3	10,3	10,2	10,1	10,1	+0,55
4	48	♂	A	9,6	9,9	10,0	9,8	9,7	9,6	+1,12
5	62	♂	A	9,9	10,3	10,1	10,0	9,9	9,9	+0,80
6	25	♀	A	9,6	10,0	9,9	9,9	9,8	9,7	+1,50
7	50	♀	A	9,5	9,7	9,9	9,7	9,6	9,6	+1,10
8	51	♀	A	10,0	10,3	10,2	10,2	10,1	10,1	+1,02
9	59	♀	A	9,4	9,5	9,6	9,6	9,5	9,4	+0,57
10	60	♀	A	10,0	10,3	10,3	10,0	10,0	10,1	+0,77
			$\overline{Ca_t} - \overline{Ca_0}$ =	*0,0*	*+0,3*	*+0,3*	*+0,1*	*+0,1*	*0,0*	$\bar{F}_3 = +0,37$

Tabelle 4. *Calciumcitratverbindung bei zehn normaciden Versuchspersonen*

Lfd. Nr.	Alter in Jahren	Geschlecht	FA	Ca_t = Calcium in mg-%						
				Nüchtern	1½ Std	2½ Std	3½ Std	4½ Std	6½ Std	F
1	21	♂	N	10,2	10,9	11,0	11,0	10,7	10,3	+3,32
2	22	♂	N	10,7	11,4	11,8	11,5	11,5	11,1	+4,32
3	23	♂	N	10,9	11,8	12,2	12,3	11,7	11,3	+5,42
4	24	♂	N	11,2	11,8	12,4	12,2	11,7	11,2	+3,70
5	24	♂	N	9,6	10,3	10,5	10,4	10,6	10,1	+4,57
6	25	♂	N	10,6	11,2	11,8	12,2	11,9	11,5	+6,40
7	27	♂	N	10,5	11,1	11,7	11,4	11,0	11,1	+4,30
8	46	♂	N	10,5	10,9	11,3	11,8	11,6	11,0	+4,75
9	30	♀	N	9,3	9,6	10,0	10,5	10,4	9,7	+4,32
10	30	♀	N	10,0	10,7	11,2	11,4	11,6	10,7	+6,57
			$\overline{Ca_t} - \overline{Ca_0}$ =	*0,0*	*+0,6*	*+1,0*	*+1,1*	*+0,9*	*+0,5*	$\bar{F}_4 = +4,77$

Lfd. Nr.	Alter in Jahren	Geschlecht
1	47	[illegible]
2	68	[illegible]
3	30	[illegible]
4	34	[illegible]
5	55	[illegible]
6	57	[illegible]
7	62	[illegible]
8	64	[illegible]
9	51	[illegible]
10	68	[illegible]
11	57	[illegible]
12	57	[illegible]
13	65	[illegible]

Erg

Die Ergebnisse unsere
bis 5 und Abb. 1—3 wie
nung der Flächen F als
tatsächliche Kurvenverla
durch einen angenähert
schen je 2 Meßpunkten e
tion Ca_t angenommen
Flächenstücke auf diese
Von den Durchschnitts
tration zur Zeit t sind d
so daß $\overline{Ca_t} - \overline{Ca_0}$ die du
gen gegenüber dem Aus

Abweichungen in de
schnitten der Gruppen
der Zahlen für $\overline{Ca_t} - \overline{Ca_0}$.

Die Berechnung der
Mittelwertes der Flächen

$$s\bar{F} = \Big[$$

Für die einzelnen Ver
folgende Werte:

1. 10 Kontrollpersonen (Nüchternschwankung)
2. 10 gesunde Normacide, getestet mit Calciumcarbonat
3. 10 histaminrefraktär Anacide, ebenfalls mit Calciumcarbonat
4. 10 gesunde Normacide, die die oben erwähnte Calciumcitrat-Verbindung erhielten
5. 13 histaminrefraktär Anacide, ebenfalls mit Calciumcitrat
6. Bei 25 Normaciden betru die Nüchtern-Calcium-Konzentration im Serum (Ausgangswerte)
7. Bei 28 histaminrefraktär anaciden Patienten war d Nüchternwert (Ausgangswert) dagegen

Die Prüfung der Dif
schon ausgeführt — du

$c =$

Tabelle 5. *Calciumcitratverbindung bei 13 histaminrefraktär Anaciden*

Lfd. Nr.	Alter in Jahren	Geschlecht	FA	Ca_t = Calcium in mg-%						
				Nüchtern	1½ Std	2½ Std	3½ Std	4½ Std	6½ Std	F
1	47	♂	A	9,1	9,8	10,0	9,9	9,9	9,4	+4,17
2	68	♂	A	8,9	9,9	10,3	9,9	9,6	9,3	+5,85
3	30	♀	A	9,9	10,6	10,9	10,7	10,5	10,0	+3,67
4	34	♀	A	10,0	10,6	10,8	10,6	10,5	10,3	+3,20
5	55	♀	A	10,8	11,3	11,6	11,6	11,1	10,8	+2,67
6	57	♀	A	10,2	10,8	11,3	11,1	10,7	10,2	+3,50
7	62	♀	A	9,6	10,2	10,5	10,6	10,3	10,0	+4,10
8	64	♀	A	9,5	9,9	10,7	10,5	10,1	9,9	+4,00
9	51	♂	A	10,0	10,1	10,3	10,2	10,0	10,0	+0,62
10	68	♂	A	9,9	10,3	10,3	10,2	10,1	9,9	+1,50
11	57	♀	A	10,0	10,1	10,2	10,0	9,9	9,7	−0,10
12	57	♀	A	10,2	10,4	10,5	10,4	10,3	10,2	+0,87
13	65	♀	A	10,1	10,5	10,6	10,3	10,4	10,1	+1,65
			$\overline{Ca}_t-\overline{Ca}_0=$	*0,0*	*+0,5*	*+0,8*	*+0,6*	*+0,4*	*+0,1*	$\overline{F}_5=+2,75$

Ergebnisse

Die Ergebnisse unserer Versuche sind in Tabelle 1 bis 5 und Abb. 1—3 wiedergegeben. Bei der Berechnung der Flächen F als Resorptionsmaß mußte der tatsächliche Kurvenverlauf aus begreiflichen Gründen durch einen angenäherten ersetzt werden, indem zwischen je 2 Meßpunkten ein linearer Verlauf der Funktion Ca_t angenommen wurde und die betreffenden Flächenstücke auf diese Weise Trapeze darstellen. Von den Durchschnittswerten der Calcium-Konzentration zur Zeit t sind die Ausgangswerte abgezogen, so daß $\overline{Ca}_t-\overline{Ca}_0$ die durchschnittlichen Veränderungen gegenüber dem Ausgangswert darstellt.

Abweichungen in den Werten F bei den Durchschnitten der Gruppen erklären sich durch Runden der Zahlen für $\overline{Ca}_t-\overline{Ca}_0$.

Die Berechnung der mittleren Abweichung des Mittelwertes der Flächen $\overline{F}$ erfolgte wie üblich nach:

$$s_{\overline{F}} = \sqrt{\frac{\sum (F-\overline{F})^2}{n\,(n-1)}}$$

Für die einzelnen Versuchsgruppen erhielten wir so folgende Werte:

1. 10 Kontrollpersonen (Nüchternschwankung) $\overline{F}_1 = -0{,}25 \pm 0{,}14$ mg-% × Std
2. 10 gesunde Normaciden, getestet mit Calciumcarbonat $\overline{F}_2 = +4{,}47 \pm 0{,}30$ mg-% × Std
3. 10 histaminrefraktär Anaciden, ebenfalls mit Calciumcarbonat $\overline{F}_3 = +0{,}87 \pm 0{,}11$ mg-% × Std
4. 10 gesunde Normaciden, die die oben erwähnte Calciumcitrat-Verbindung erhielten $\overline{F}_4 = +4{,}77 \pm 0{,}34$ mg-% × Std
5. 13 histaminrefraktär Anaciden, ebenfalls mit Calciumcitrat $\overline{F}_5 = +2{,}75 \pm 0{,}48$ mg-% × Std
6. Bei 25 Normaciden betrug die Nüchtern-Calcium-Konzentration im Serum (Ausgangswerte) $\overline{Ca}_{0N} = 10{,}4 \pm 0{,}10$ mg-%
7. Bei 28 histaminrefraktär anaciden Patienten war der Nüchternwert (Ausgangswert) dagegen $\overline{Ca}_{0A} = 9{,}5 \pm 0{,}10$ mg-%

Die Prüfung der Differenzen erfolgte — wie oben schon ausgeführt — durch Berechnung von

$$c = \frac{D}{s_D},$$

D ist die Differenz der zu vergleichenden Mittelwerte. s_D stellt die mittlere Abweichung der Mittelwertdifferenz dar:

$$s_D = \sqrt{s^2_{\bar{x}_1} + s^2_{\bar{x}_2}},$$

wobei $\bar{x}$ allgemein ein arithmetisches Mittel bedeutet.

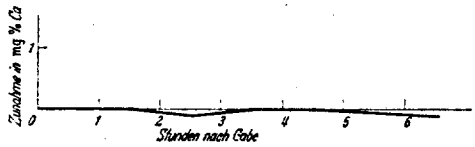

Abb. 1. Durchschnittliche Calcium-Nüchternschwankung im Serum bei 10 Kontrollpersonen (nach Tabelle 1)

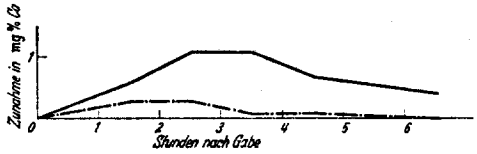

Abb. 2. 25 mg Ca/kg als Calciumcarbonat oral. Durchschnittliche Calcium-Konzentrationserhöhungen im Serum bei 10 Normaciden ——— (nach Tabelle 2) und bei 10 hist. refr. Anaciden —·—·— (nach Tabelle 3)

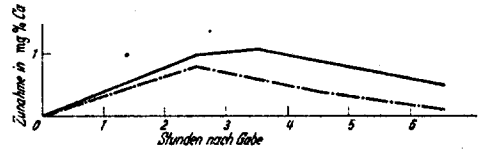

Abb. 3. 25 mg Ca/kg als Calciumcitrat-Verbindung oral. Durchschnittliche Calcium-Konzentrationserhöhungen im Serum bei 10 Normaciden ——— (nach Tabelle 4) und bei 13 hist. refr. Anaciden —·—·— (nach Tabelle 5)

So berechnet sich bei folgenden Vergleichen:

Mittelwerte	D	c	Mittelwerte	D	c
$\overline{F}_2-\overline{F}_1$	+4,72	*14,0*	$\overline{F}_2-\overline{F}_3$	+3,60	*11,2*
$\overline{F}_3-\overline{F}_1$	+1,12	*6,6*	$\overline{F}_4-\overline{F}_5$	+2,02	*3,4*
$\overline{F}_4-\overline{F}_1$	+5,02	*13,9*	$\overline{F}_5-\overline{F}_2$	+1,88	*3,9*
$\overline{F}_5-\overline{F}_1$	+3,00	*6,0*	$\overline{Ca}_{0N}-\overline{Ca}_{0A}$	+0,9	*6,4*
$\overline{F}_4-\overline{F}_2$	+0,30	0,7			

Diskussion

Um eine bestimmte Aussage über die Calcium-Konzentrationserhöhung im Serum nach oraler Kalkgabe machen zu können, mußten wir zunächst die

Größe der physiologischen Schwankung des Calcium-Gehaltes im menschlichen Serum während der Versuchsdauer ermitteln. Leider existieren hierüber in der Literatur nur wenig Angaben.

So fand VOLLMER[86] während einer 6stündigen Versuchsdauer in 4 Versuchen maximale Abweichungen von 0,5 mg-%. SIWE[79] berichtet bei 8 3stündigen Versuchen über maximale Veränderungen von 1,0 mg-% und KIRCHER[14] erhielt bei 34 Kindern als durchschnittliche Differenz zwischen 2 Blutabnahmen innerhalb von $1^1/_2$—2 Std 0,55 mg-% Calcium. Bei 7 Kindern war die Differenz allerdings größer als 1 mg-%.

Da diese Angaben für unsere Zwecke nicht ausreichten, waren wir auf eigene Untersuchungen angewiesen. Wie Tabelle 1 zeigt, wurden bei 10 Kontrollpersonen maximale Konzentrationsschwankungen zwischen 0,1—0,7 mg-% beobachtet. Die durchschnittlichen Veränderungen gegenüber dem Ausgangswert (Ca_t—Ca_0) weichen jedoch bei den einzelnen Entnahmezeiten nicht mehr als 0,1 mg-% voneinander ab, so daß hiermit eine ausreichende Grundlage für die folgenden statistischen Vergleiche gegeben war. Der Mittelwert der Flächen beträgt $-0{,}25 \pm 0{,}14$ mg-% × Std und seine Abweichung von 0 liegt sogar innerhalb der Zufallsstreuung.

Wie oben schon erwähnt, beträgt die Fehlerbreite der angewandten komplexometrischen Calcium-Bestimmungsmethode im Serum nur $\pm 0{,}1$ mg-%[69]. Außerdem wurden immer Doppelbestimmungen ausgeführt. Da mögliche Meßfehler sich durch Verwendung der Mittelwerte der Konzentrationsveränderungen bei 10 bzw. 13 Versuchspersonen entsprechend vermindern, dürfen wir methodische Fehlermöglichkeiten bei unseren Überlegungen vernachlässigen.

Bei Verwendung des Mittelwertes der Fläche als Maß für die Calcium-Resorption der betreffenden Versuchsgruppe lassen sich folgende nach oraler Gabe von 25 mg Ca/kg Körpergewicht erhaltene Resorptionskurven gegen die Nüchternschwankung als nicht zufällig abgrenzen ($c > 3$):

1. Calciumcarbonat bei gesunden Normaciden (Tabelle und Abb. 2).
2. Calciumcarbonat bei histaminrefraktär Anaciden (Tabelle 3 und Abb. 2).
3. Calciumcitrat-Verbindung bei gesunden Normaciden (Tabelle 4 und Abb. 3).
4. Calciumcitrat-Verbindung bei histaminrefraktär Anaciden (Tabelle 5 und Abb. 3).

Damit wurde bewiesen, daß die Resorption oral verabreichter Calcium-Verbindungen durch Bestimmung des Calcium-Gehaltes im Serum nachgewiesen bzw. geprüft werden kann. Hierbei blieb die Dosierung im physiologischen Bereich (70 kg schwere Person z. B. 1,75 g Calcium).

Über den rein qualitativen Resorptionsnachweis hinaus ist es durch die angewandte Methode möglich, vergleichende quantitative Aussagen bei den einzelnen Versuchsgruppen zu machen. Obwohl bei den Normaciden die Resorption der Calciumcitrat-Verbindung mit 4,77 mg-% × Std etwas größer ist als die des Calciumcarbonats (4,47), läßt sich diese Differenz statistisch nicht sichern ($c = 0{,}7$). Wir können daher bei gesunden Menschen mit normalen Magensaftwerten keinen sicheren Unterschied in der Resorption dieser beiden Calcium-Verbindungen feststellen.

Dagegen ist die Resorption beider Verbindungen bei den Patienten ohne Magensäure signifikant niedriger als bei den Normaciden, wobei noch insofern ein Unterschied besteht, als die angewandte saure Calciumcitrat-Verbindung bei den histaminrefraktär Anaciden deutlich besser resorbiert wird als das neutrale Calciumcarbonat ($D = 1{,}88$; $c = 3{,}9$).

Allerdings fällt bei der 5. Versuchsgruppe die mit fast $\pm 0{,}5$ mg-% × Std relativ große mittlere Abweichung auf. In der Tat zeigen die Patienten 1—8 dieser Gruppe einen mittleren Calcium-Resorptionswert von $+3{,}90 \pm 0{,}33$ mg-% × Std, die Versuchspersonen 9 bis 13 dagegen nur einen solchen von $+0{,}91 \pm 0{,}32$ mg-% × Std. Hier wäre evtl. an einen die Calcium-Resorption noch zusätzlich störenden Faktor wie z. B. beschleunigte Darmpassage, entzündliche oder degenerative Prozesse der Darmschleimhaut usw. zu denken.

Aus den Tabellen 2—5 und den Abb. 2 und 3 geht hervor, daß die Calcium-Resorption bei den gesunden Normaciden nach $6^1/_2$ Std offenbar noch nicht abgeschlossen ist, während die anaciden Kurven zu diesem Zeitpunkt den Ausgangswert bereits wieder erreicht haben.

Auf die Bedeutung der Magen- und Darmkrankheiten für die Calcium-Resorption ist schon wiederholt hingewiesen worden. So beobachteten u. a. HOLT u. Mitarb.[37] und später HALLMAN u. Mitarb.[26] bei chronischen Verdauungsstörungen eine verminderte Calcium-Resorption. In neuerer Zeit fanden NICOLAYSEN und RAGARD[68] bei 15 gastrektomierten Patienten eine stark negative Calcium-Bilanz.

Allerdings werden klinisch faßbare Calcium-Mangelzustände in der Regel nur dann auftreten, wenn mehrere Faktoren (chronische Calcium-Resorptionsstörung, Calcium-Mangelernährung oder erhöhter Calcium-Bedarf) bei demselben Individuum zusammentreffen.

Natürlich liegt es nahe, eine mangelhafte Calcium-Zufuhr und -Resorption mit anderen Störungen des Calcium-Stoffwechsels im Sinne „alimentärer Osteopathien"[21] in Beziehung zu bringen[1, 12, 15, 30, 51, 61–65, 70, 71, 78, 86, 87]. Insbesondere ist schon des öfteren auf die Bedeutung der Anacidität bei der Entstehung resorptionsbedingter calcipenischer Osteopathien im Sinne von BARTELHEIMER und SCHMITT-ROHDE[5] hingewiesen worden:

Bereits 1938 prägte MEULENGRACHT[64] hierfür den Begriff der „Osteomalacia achylica". Seine Untersuchungen sind später u. a. von PAHLKE[72] ergänzt worden und erst in neuester Zeit hat FRANZ[24] den Zusammenhängen zwischen Salzsäuremangel im Magensaft und der Entstehung calcipenischer Osteopathien eine ausführliche Studie gewidmet.

Durch den mit unserer Arbeit erbrachten Nachweis der gestörten Resorption besonders neutraler Calcium-Verbindungen beim Salzsäuremangel des Magens kann die Pathogenese dieser Krankheitsbilder wie folgt ergänzt werden:

HCl-Defizit des Magens → verminderte Calcium-Resorption → resorptionsbedingte calcipenische Osteopathien.

Wenn man dazu noch bedenkt, daß im Alter eine Abnahme der Magen-Salzsäure physiologisch ist (zusammenfassende Übersicht in neuester Zeit bei RITTER[73]), so ergibt sich aus der daraus resultierenden mangelhaften Calcium-Resorption zumindest ein wichtiger ätiologischer Faktor für die Entstehung der altersbedingten Osteopathien.

Die gleichwohl geri
Differenz der mittlere
tration im Serum (Nüch
aciden (10,4 mg-%) und
(9,5 mg-%) gewinnt bei
Durchschnittsalters der
über 27,2 Jahre bei den
Nach Untersuchungen
NOWSKI[18] an etwa 170 ge
klassen ist nämlich eher
Konzentration mit den
Obwohl also der Blutk
funktion des Knochen
werden muß (APITZSC
ROHDE[82]), kommt es
Resorptionsstörungen u
nischen Calcium-Mangel
niedrigung des Serum-C

Zusammenfassung. B
die intestinale Calcium-
von 25 mg Calcium je
stimmungen des Calcium
Zu diesem Zweck wurde
gebildet:

1. 10 Kontrollperson
schwankung der Calciu
während der Versuchsda
2. 10 gesunde Mensch
werten, bei denen Calciu
diente.
3. 10 histaminrefrak
denen ebenfalls Calcium
4. 10 gesunde Norma
Calciumcitrat-Verbindung
5. 13 histaminrefrak
Resorption gleichfalls m
bindung geprüft wurde.

Als Maß für die Grö
wir die Fläche der zugeh
mg-% × Std. Die Versu
Patienten durchgeführt.
in allen Fällen $6^1/_2$ Std.

Das Calcium im Ser
bestimmt.

Die Ergebnisse wurde
Unsere Versuche zeig
1. Die Resorption
Verbindungen kann durc
Gehaltes im Serum nachg
2. Von den beiden
Calcium-Verbindungen ze
mit normalen Magensaf
Verbindung einen besser
ließ sich die Differenz zum
nicht sichern.
3. Dagegen ist die Res
bei den Patienten ohne
niedrigt, wobei die angev
Verbindung bei diesen M
resorbiert wird als das n
4. Die nüchterne
Serum ist bei der ana
9,5 mg-% signifikant n
Gehalt bei den Normacid

Literatur. 1 ADAMS, M., SNELL: Amer. J. Physiol. 1

Die gleichwohl geringe, wenn auch signifikante Differenz der mittleren Calcium-Ausgangskonzentration im Serum (Nüchternwert) zwischen der normaciden (10,4 mg-%) und der anaciden Versuchsgruppe (9,5 mg-%) gewinnt bei Berücksichtigung des höheren Durchschnittsalters der 2. Gruppe (48,6 Jahre gegenüber 27,2 Jahre bei den Normaciden) an Bedeutung. Nach Untersuchungen von ELKINTON und DANOWSKI[19] an etwa 170 gesunden Personen aller Altersklassen ist nämlich eher eine Zunahme der Calcium-Konzentration mit dem Lebensalter zu erwarten. Obwohl also der Blutkalkgehalt durch die Depotfunktion des Knochengewebes konstant gehalten werden muß (APITZSCH[4], JESSERER[41], SCHMITT-ROHDE[82]), kommt es bei chronischen Calcium-Resorptionsstörungen und dadurch bedingtem chronischen Calcium-Mangel zu einer signifikanten Erniedrigung des Serum-Calciums.

Zusammenfassung. Bei 53 Versuchspersonen wurde die intestinale Calcium-Resorption nach oraler Gabe von 25 mg Calcium je kg Körpergewicht durch Bestimmungen des Calcium-Gehaltes im Serum geprüft. Zu diesem Zweck wurden folgende Versuchsgruppen gebildet:

1. 10 Kontrollpersonen, bei denen die Nüchternschwankung der Calcium-Konzentration im Serum während der Versuchsdauer ermittelt wurde.

2. 10 gesunde Menschen mit normalen Magensaftwerten, bei denen Calciumcarbonat als Testsubstanz diente.

3. 10 histaminrefraktär anacide Patienten, bei denen ebenfalls Calciumcarbonat verwendet wurde.

4. 10 gesunde Normacide, die eine handelsübliche Calciumcitrat-Verbindung („Dago C") erhielten.

5. 13 histaminrefraktär Anacide, bei denen die Resorption gleichfalls mit dieser Calciumcitrat-Verbindung geprüft wurde.

Als Maß für die Größe der Resorption benutzten wir die Fläche der zugehörigen Kurve, ausgedrückt in mg-% × Std. Die Versuche wurden an nüchternen Patienten durchgeführt. Die Versuchsdauer betrug in allen Fällen $6^1/_2$ Std.

Das Calcium im Serum wurde komplexometrisch bestimmt.

Die Ergebnisse wurden statistisch verglichen.

Unsere Versuche zeigen:

1. Die Resorption oral verabreichter Calcium-Verbindungen kann durch Bestimmung des Calcium-Gehaltes im Serum nachgewiesen bzw. geprüft werden.

2. Von den beiden zur Anwendung gelangten Calcium-Verbindungen zeigte bei gesunden Menschen mit normalen Magensaftwerten die Calciumcitrat-Verbindung einen besseren Resorptionswert, jedoch ließ sich die Differenz zum Calciumcarbonat statistisch nicht sichern.

3. Dagegen ist die Resorption beider Verbindungen bei den Patienten ohne Magensäure signifikant erniedrigt, wobei die angewandte saure Calciumcitrat-Verbindung bei diesen Menschen noch deutlich besser resorbiert wird als das neutrale Calciumcarbonat.

4. Die nüchterne Calcium-Konzentration im Serum ist bei der anaciden Versuchsgruppe mit 9,5 mg-% signifikant niedriger als der Calcium-Gehalt bei den Normaciden (10,4 mg-%).

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Der p-Phenylendiamin(PPD)-Oxydasetest als eine Laboratoriumsmethode zur Diagnostik der Hyperthyreose

Von

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Hahn und Keler-Bačoka veröffentlichten kürzlich Resultate von Kupferbestimmungen im Serum bei 46 Hyperthyreosekranken[1,2]. Die Autoren fanden bei dieser Krankheit eine erhöhte Serumkupferkonzentration, was mit den wenigen auf diesem Gebiete veröffentlichten Publikationen[3] im Einklang steht.

Bekanntlich ist über 90% des Serumkupfers als Cofaktor an das Enzym Coeruloplasmin gebunden[4], so daß Serumkupfer- und Coeruloplasminkonzentrationen beim Gesunden und bei verschiedenen Krankheiten parallel gehen[5,6]. Erhöhte Kupfer- oder Coeruloplasminkonzentrationen im Serum werden auch in der Schwangerschaft[4,5], bei malignen Tumoren und chronischen Infekten[4,5], bei Schizophrenie[7] und anderen Geisteskrankheiten[8] sowie bei Leberkrankheiten[9] gefunden.

Das Enzym Coeruloplasmin ist durch eine katalytisch-oxydative Aktivität gegenüber verschiedenen Substraten, besonders den Lösungen des p-Phenylendiamins (PPD) und seines Dimethylsalzes (DDP) gekennzeichnet. Deswegen wird das Enzym auch PPD- oder DDP-Oxydase bezeichnet. Die Bestimmung der oxydativen Coeruloplasminaktivität kann mittels Substratlösungen dieser beiden Salze vorgenommen werden[6,10,11].

Nach Lerner[12] beruht die physiologische Aufgabe des Kupferproteids darin, den oxydativen Abbau der Aminosäure Tyrosin (p-Oxyphenylalanin), des Bausteines des Schilddrüsenhormons Thyroxin, über das Dioxyphenylalanin (DOPA) und dessen Chinonderivat (DOPA-Chinon) zu katalysieren. Aus diesem Grunde wird das kupferbindende Enzym auch Tyrosinase und DOPA-Oxydase genannt.

Bekanntlich sind die Oxydationsprozesse bei der Hyperthyreose allgemein beschleunigt. Im Anschluß an unsere bisherigen Beobachtungen[1,2,6] haben wir daher bei 31 unbehandelten Hyperthyreosekranken parallel zum Grundumsatz die Oxydationsaktivität des Serumcoeruloplasmins mittels des PPD-Oxydasetestes sowie das Serumkupfer bestimmt. Weiterhin haben wir die gleichen Untersuchungen bei verschiedenen Patienten mit Hyperthyreose vor und während der Therapie mit Thyreostatica der Thiouracilgruppe* durchgeführt.

* Metiltiouracil „Pliva" Zagreb (1 Tablette = 0,01 g); Propycil (Propylthiouracil) „Kali-Chemie" Hannover (1 Tablette = 0,05 g).

Material und Methoden

Es wurden frische, klare Seren von unbehandelten, klinisch diagnostizierten 31 Hyperthyreosekranken auf die PPD-Oxydaseaktivität und die Serumkupferkonzentration untersucht. Gleichfalls wurde der Grundumsatz der Patienten bestimmt. Die PPD-Oxydaseaktivität wurde nach der eigenen Modifikation[6] der Methode nach Goldschmidt[11] bestimmt.

Das Prinzip besteht darin, daß eine auf pH 7,2 gepufferte p-Phenylendiaminhydrochloridlösung durch die PPD-Oxydaseaktivität des Serums oxydiert wird. Die optische Dichte der oxydierten Lösung wird elektrophotometrisch gemessen und nach Abzug des Leerwertes auf Extinktionseinheiten umgerechnet.

Reagentien. 1. 0,066 M isotonische Pufferlösung pH 7,2 nach Soerensen. 2. p-Phenylendiaminhydrochloridlösung 0,10% frisch bereitet. 3. Physiologische Kochsalzlösung.

Ausführung. Die Untersuchungen werden im Wasserbade bei 37°C ausgeführt. In einer Gläschenreihe (*A*), entsprechend der Zahl der Untersuchungen, werden zuerst die Serumfarbenkompensationsversuche vorbereitet, bestehend aus 2 ml der Pufferlösung 1 und 0,20 ml Serum. Je 2 ml der Lösung 2 werden in die zweite Gläschenreihe (*B*) gemessen und vor der ersten und nach der letzten Untersuchung wird je ein Blindversuch, der aus 2 ml der Lösung 2 und 0,20 ml der Lösung 3 besteht, vorbereitet. Sofort anschließend wird in die Gläschenreihe *B* 0,20 ml Serum im Zeitabstand von 30 sec zugegeben, und genau 5 min nach der ersten Serumzugabe werden im Zeitabstand von 30 sec die optischen Dichten der einzelnen Lösungen (*B*) nacheinander am Zeiss ELKO II-Photometer mit Filter 535 mμ und bei Schichtdicke 0,50 cm gemessen. Die Kompensationsversuche der ersten Gläschenreihe (*A*) dienen hierbei zur Kompensation der Serumfarbe. Vor und nach den Serumlösungen werden die Blindversuche, welche kein Serum enthalten, gegen die Lösung 3 gemessen.

Berechnung. Der Mittelextinktionswert der zwei Blindversuche wird vom Extinktionswert des Serums abgezogen und mit 1000 multipliziert (PPD-Oxydase-Einheit).

Das Serumkupfer wurde nach Heilmeyer u. Mitarb.[3] bestimmt.

Normalwerte. PPD-Oxydaseaktivität: 20—60 PPD-Einheiten mit dem Mittelwert 38,64 und einer Standardabweichung von ±11,45. Serumkupfer: 81—140 μg-%; Mittelwert 120,20, Standardabweichung ±16,35.

Grundumsatz: Streuung ±10%.

Alle biochemischen Untersuchungen wurden in Doppelbestimmungen ausgeführt.

Versuchsanordnung und Ergebnisse

Die Befunde von PPD-Oxydaseaktivität und Serumkupfergehalt sowie die Grundumsatzwerte bei den 31 unbehandelten Hyperthyreosekranken sind in der Tabelle dargestellt.

Die Resultate der Tabelle zeigen eine grundsätzliche Übereinstimmung der PPD-Oxydaseaktivität mit den Serumkupferwerten: unter 31 untersuchten

Tabelle. *Darstellung von Ergebni Grundstoffumsatzbestimmungen b*

Serum-Nr.	PPD-Oxydase E
1	81
2	61
3	85
4	98
5	80
6	78
7	88
8	86
9	62
10	51
11	51
12	87
13	86
14	113
15	65
16	56
17	96
18	89
19	63
20	59
21	58
22	121
23	60
24	78
25	83
26	63
27	76
28	63
29	94
30	67
31	31
Mittelwert	75,12
Standard-deviation	±19,27
P-Wert	<0,001

Seren wurde in 25 Seren aktivität und in 27 Fälle gefunden; die Ergebnisse b normal. Der Grundumsatz über die Normalgrenze er Oxydaseaktivität und Kup seren ist nach dem *t*-Test s <0,01.

Daraus kann geschloss daseuntersuchungen — be logischer Zustände, welche Oxydaseaktivität verursa gnostik unbehandelter Hy den können.

In anschließenden Unt schiedenen Hyperthyreos der Therapie mit Thyreos der PPD-Oxydasetest. Grundumsatz bestimmt. gebnisse dieser Untersuch vor und während der Th (300 mg pro Tag) wieder

Nach diesen Befunde 300 mg Methylthiouracil kung der PPD-Oxydase kupferkonzentration. De gleichen Zeitpunkt abzu klinische Anzeichen der bis zur Genesung des Pa anderen biochemischen A werte abzusinken.

MINERAL STUDIES IN OVINE PHOSPHATIC UROLITHIASIS[1]

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AMONG the many factors implicated in the occurrence of urolithiasis in ruminant and monogastric animals are those related to dietary mineral imbalance and abnormalities of mineral metabolism. Magnesium, calcium, phosphorus and potassium frequently have been associated with the formation of urinary calculi. Coburn and Packett (1962) produced oxalate stone in weanling rats on a low phosphorus (0.16%) diet with a wide Ca:P ratio. Robbins *et al.* (1965) produced a high incidence of calculi in rats by feeding a specific mineral mix which supplied 1.20% phosphorus to the diet. Vitamin B_6 deficiency increased the incidence of renal stones (Faragalla and Gershoff, 1963), but Van Reen *et al.* (1964) believe that sulfate groups resulting from methionine metabolism reduce stone formation. Haag and Palmer (1928) found phosphatic calculi in rats receiving rations low in $CaCo_3$ and high in $MgCO_3$ and H_3PO_4. They pointed to high magnesium as a disturbing factor in nutrition. Cunningham and Cunningham (1938) prevented the formation of bladder calculi in rats on a high calcium diet by the addition of magnesium. Watchorn (1932) reported that rats fed a diet containing an excess of $MgCO_3$ developed urinary calculi in many cases.

A number of workers have produced experimental urolithiasis in ruminants and studied mineral changes after unusually high dietary additions of sodium or potassium phosphate (Elam *et al.*, 1956; Cornelius *et al.*, 1959; Butcher, 1961; Emerick and Embry, 1963; Bushman *et al.*, 1965).

Urolithiasis is a major cause of death in feeder steers and wethers on high-energy fattening rations. Packett *et al.* (1958) reported a fattening ration consisting of natural ingredients and balanced with respect to calcium and phosphorus content but extremely calculogenic. Numerous studies using this diet or modifications have established its calculogenic nature and elucidated changes with respect to phosphorus and magnesium metabolism (Crookshank and Robbins, 1962; Packett and Hauschild, 1964; Kunkel *et al.*, 1961; Crookshank *et al.*, 1965, 1967; Packet and Butcher, 1963). Udall (1959) also induced a high incidence of calculi using fattening diets balanced with respect to calcium and phosphorus. The work of Black *et al.* (1947) implicates low dietary calcium as a critical factor in ruminant urolithiasis.

Using modifications of the experimental calculogenic diet of Packett and Hauschild (1964), the influence of sodium citrate, calcium citrate, vitamin D and calcium carbonate on development of urolithiasis was investigated. Patterns of serum mineral levels and mineral balance were studied to determine the etiology of calculi development under feedlot conditions.

Experimental

A total of 142 wether feeder lambs was used in two separate experiments. Newly arrived Texas lambs were fed alfalfa hay with free access to salt and water for one week prior to the initial collection of blood and placing on experimental diets. In experiment I, 82 wethers weighing approximately 36 kg. each were divided into four groups (groups 1-4) and were fed the pelleted diet shown in table 1. The diets were made available in a self-feeder along with salt and water free choice.

Blood samples were drawn from the jugular vein at 0, 3, 7 and 11 wk. to follow blood mineral changes during the course of calculi development. Four-day urine collections were made from five sheep of each group between the eighth and 12th weeks. Sodium and potassium as well as calcium and magnesium were analyzed by methods described by Willis (1960, 1961). Serum inorganic phosphorus was determined by an adaptation of the method of Fiske and Subbarow (1925). Total urine phosphorus was analyzed as described by Hawk *et al.* (1954). Daily urine volumes, pH, and specific gravity measurements also were determined for urine samples.

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TABLE 1. COMPOSITION OF DIETS IN EXPERIMENTS I AND II

Diet components	Experiment I				Experiment II		
	1 Basal	2 Sodium citrate	3 Calcium citrate	4 Vitamin D_3	5 Basal	6 Calcium citrate	7 Calcium carbonate
Ingredients, %							
Sorghum grain	45	42	42	45	45	42	45
Cottonseed hulls	27	27	27	27	27	27	27
Cotton seed meal	10	10	10	10	10	10	10
Alfalfa	8	8	8	8	8	8	8
Molasses	10	10	10	10	10	10	10
Sodium citrate	..	3[a]	..	..	..	..	..
Calcium citrate	..	..	2.95[b]	..	..	2.95[b]	..
Vitamin D_3	..	..	..	..[c]	..	..	..
Iodized salt[d]	..	..	..	..	0.5	0.5	0.5
Calcium carbonate[e]	..	..	..	..	..	..	1.58[e]
Mineral composition, %							
Calcium	0.30	0.30	0.30	0.30	0.36	0.87	0.80
Magnesium	0.22	0.22	0.22	0.22	0.22	0.21	0.21
Phosphorus	0.30	0.30	0.30	0.30	0.31	0.30	0.31
Sodium	..[f]	..[f]	..[f]	..[f]	0.33	0.33	0.33
Potassium	1.14	1.14	1.14	1.14	1.14	1.14	1.14

[a] Sodium citrate supplied by Chas. Pfizer and Co., Terre Haute, Indiana.
[b] Calcium citrate purified by Chas. Pfizer and Co., Terre Haute, Indiana.
[c] Vitamin D_3 of feed, 440 I.U./kg. of feed, supplied by Nopco Chemical Co., Harrison, N. J.
[d] Iodized sodium chloride *ad libitum* in Experiment I.
[e] Calcium carbonate, supplied by Chas. Pfizer and Co., Terre Haute, Indiana.
[f] Sodium chloride *ad libitum*.

In experiment II, 60 wethers were divided into three groups, groups 5, 6 and 7. Group 5 and 6 each consisted of 25 sheep; group 7 of 10 sheep. In contrast to groups 1–4 (experiment I) where NaCl was offered *ad libitum*, groups 5, 6 and 7 (experiment II) were fed 0.5% NaCl blended into the pellets. Udall (1962) reported that a diet containing 4% or greater NaCl inhibited the formation of uroliths. The 0.5% level of NaCl was not intended to inhibit calculi formation, but was added to control the sodium intake and, thereby, make balance studies possible. Blood samples were taken initially and at the end of 3 and 7 weeks. Serum calcium and magnesium were analyzed by atomic absorption spectroscopy as described above but serum sodium and potassium were not determined.

Nine wethers each from groups 5 and 6 were placed in metabolism cages and 5-day balance studies were made on calcium, magnesium, phosphorus, sodium and potassium. Each sheep was fed 2 lbs. of ration per day while deionized water was available *ad lib.* Urine was collected and measured daily and portions were saved for analyses. Feces were dried, weighed and ground. Representative samples of the feces from each wether were then ashed, dissolved in acid, and diluted to an appropriate volume.

The incidence of calculi was determined by examination of the kidneys, bladder and urethrae of those wethers which died during the experiments and of the remaining wethers which were sacrificed at the termination of the experiments. Data were analyzed statistically by the use of analysis of variance as described by Snedecor (1956).

Results and Discussion

In both experiments I and II, in the highly calculogenic groups 1, 2, 4, 5 and 7, serum phosphorus and magnesium levels were raised, whereas serum calcium gradually declined (table 2). At the end of 3 wk., serum phosphorus and magnesium levels were significantly increased ($P<.05$) and, except for groups 1 and 3, serum calcium levels dropped significantly ($P<.05$). Group 3, the calcium citrate group with 0% calculi incidence, had significantly lower serum magnesium ($P<.05$) and lower serum phosphorus ($P<.01$) at 7 and 11 weeks. Calcium levels within this non-calculogenic group were higher ($P<.05$) than in all other groups at 3 and 11 wk. and also higher than group 4 at 7 weeks. The serum magnesium of the highly calculogenic vitamin D_3 group (Group 4, 80% calculi incidence) was increased ($P<.01$) at 7 and 11 wk. over the lower serum magnesium of the non-calculogenic calcium citrate group. It is clear that

calcium citrate is the non-calculogenic substance. The addition of citrate as sodium citrate (group 2) or calcium as calcium carbonate (group 7) did not prevent the occurrence of calculi nor alter the serum magnesium nor phosphorus levels. The ability of calcium citrate to raise the calcium and to lower the phosphorus and magnesium levels resulted in lower P/Ca and Mg/Ca ratios and prevented the formation of calculi.

Although serum sodium and serum potassium levels increased in all groups during the experiment, there were no significant differences between the calculi and non-calculi groups.

The unusually high phosphatic calculi incidence (80%) of wethers in group 4 (vitamin D_3 supplemented) was unexpected. Vitamin D was expected to enhance calcium absorption and alter mineral metabolism. Coburn and Packett (1962) had previously shown that the addition of vitamin D_3 to a calculogenic diet for monogastric animals (rats) reduced the occurrence and severity of calcium oxalate urolithiasis. The wethers of group 4 had significantly higher serum magnesium and lower serum calcium at the end of 7 wk. but phosphorus levels were elevated as in other calculogenic groups. Kunkel *et al.* (1961) also found that incidence of urinary calculi in

TABLE 2. CALCULI INCIDENCE AND SERUM CALCIUM, MAGNESIUM, PHOSPHORUS, SODIUM AND POTASSIUM OF WETHERS AS INFLUENCED BY DIET AND LENGTH OF EXPERIMENT

	Experiment I				Experiment II[a]		
Group	1	2	3	4	5	6	7
Treatment	Basal	Sodium citrate	Calcium citrate	Vitamin D_3	Basal	Calcium citrate	Calcium carbonate
No. of wethers	26	26	15	15	25	25	10
Incidence of gross calculi, %	56	50	0	80	56	4	70
Week							
			Serum calcium, mg./100 ml.				
0	12.9	12.4	12.2	12.3	11.6	11.6	11.8
3	12.0	11.5[b]	14.3[b, c]	10.3[b]	10.2[b]	12.6[b]	11.0[b]
7	10.5	10.9	11.3[d]	9.5	10.4	12.4	11.5
11	10.8	9.7	12.2[e]	10.4	..	..	..
			Serum magnesium, mg./100 ml.				
0	2.5	2.2	2.4	2.4	2.7	2.6	2.4
3	4.0[b]	3.0[b]	3.5[b]	3.0[b]	3.2[b]	2.6	2.8[b]
7	2.9	3.0	2.4[f]	3.8[g]	3.7	2.5	2.8
11	3.4	3.5	2.5[h]	3.6	..	..	..
			Serum phosphorus, mg./100 ml.				
0	7.0	6.9	7.9	7.9	7.0	7.1	7.3
3	11.8[b]	12.3[b]	6.7[b, i]	12.9[b]	10.7[j]	5.0	10.6[j]
7	9.9	13.1	7.0[i]	10.7	11.1[j]	6.3	10.9[j]
11	10.4	12.2	8.1[i]	10.7	..	..	..
			Serum sodium, m.Eq./l.				
0	142	142	138	136	..	..	..
3	140	137	143	146	..	..	..
7	142	153	144	139	..	..	..
11	147	149	148	147	..	..	..
			Serum potassium, m.Eq./l.				
0	5.1	5.1	5.0	5.4	..	..	..
3	4.8	5.6	5.0	4.8	..	..	..
7	4.6	5.5	5.1	5.0	..	..	..
11	5.5	5.7	5.4	5.6	..	..	..

[a] Groups 5, 6 and 7 contain the supplements plus 0.5% NaCl.
[b] Significantly different ($P<.05$) from 0 weeks.
[c] Significantly different ($P<.05$) from groups 1, 2, 4, 5, 6 and 7 at 3 weeks.
[d] Significantly different ($P<.05$) from group 4 at 7 weeks.
[e] Significantly different ($P<.05$) from groups 1, 2 and 4 at 11 weeks.
[f] Significantly different ($P<.05$) from groups 1, 2, 4, 5 and 7 at 7 weeks.
[g] Significantly different ($P<.01$) from groups 1, 2, 3, 6 and 7 at 7 weeks.
[h] Significantly different ($P<.01$) from groups 1, 2 and 4 at 11 weeks.
[i] Significantly different ($P<.01$) from groups 1, 2 and 4 at 3, 7 and 11 weeks
[j] Significantly different ($P<.01$) from initial (0 weeks) value and from group 6 values at same week.

TABLE 3. EFFECT OF DIET ON AVERAGE MINERAL COMPOSITION, VOLUME AND PROPERTIES OF URINE IN EXPERIMENT I

Group	1	2	3	4
No. of wethers	5	5	5	5
Daily urine volume, ml.	776	1800	671	1314
Urine pH	8.11	8.31	8.05	8.03
Urine specific gravity	1.037	1.026	1.053	1.021
Calcium				
Intake, mg./day	2724	2724	2724	2724
Urinary excretion, mg./100 ml.	3.4	2.1	4.2	3.0
Urinary excretion, mg./day	28.2	40.2	31.2	34.4
Magnesium				
Intake, mg./day	1998	1998	1998	1998
Urinary excretion, mg./100 ml.	62.8	37.2	46.0	62.9
Urinary excretion, mg./day	491	597	320[a]	792
Phosphorus				
Intake, mg./day	2724	2724	2724	2724
Urinary excretion, mg./100 ml.	67.6	61.2	10.6	70.0
Urinary excretion, mg./day	451	1074	85.9[b]	824
Potassium				
Intake, mg./day	10351	10351	10351	10351
Urinary excretion, mg./100 ml.	824	545	1521	451
Urinary excretion, mg./day	6585	8742	9440	5836

[a] Significantly different ($P<.05$) from group 4.
[b] Significantly different ($P<.01$) from groups 1, 2 and 4.

lambs was related to blood magnesium. Packett and Hauschild (1964) reported both high serum phosphorus and serum magnesium levels but stressed phosphorus metabolism as the more important factor in calculi development in wethers.

The effect of diet on urine volume and urine minerals is shown in table 3. Daily urine volumes were higher in the sheep on the calculogenic diets. The increase in urine volume and pH was most noticeable in the sodium citrate group. These results are in agreement with those of Packett and Butcher (1963) who also noted increased urine volume and pH upon the addition of sodium citrate to the diet of wethers without a decrease in the incidence of calculi. The data in table 4 also indicate higher daily urine volumes in sheep on calculi provoking diets. Elam *et al.* (1957) suggested that two of the reasons for the prophylactic effect of high levels of sodium chloride in the diet were increased urine volume and increased urinary acidity. The experiment of Johnson *et al.* (1940) does not support the idea that a urine of high alkalinity necessarily predisposes to calculi formation. Wethers on our non-calculogenic ration (group 3) had the lowest daily urine volume and there was no significant difference in the urine pH between the calcium citrate group (0% incidence) and the vitamin D_3 group (80% incidence).

As shown in table 3 wethers on calcium citrate supplementation (group 3) excreted significantly ($P<.01$) less phosphorus via the urine than did the calculogenic groups. Table 4 indicates a difference of 6.3% "U" between groups 5 and 6. The term "% U" is defined as the percentage of mineral excretion via the urine. Balance studies (table 5) show that not only is there a significant difference ($P<.01$) in the phosphorus balance between groups 5 and 6 but also that the wethers developing calculi excrete daily an average of 0.420 gm. less phosphorus by the fecal route and an average of 0.129 gm. more phosphorus by way of the urine. Packett and Hauschild (1964) also noted that the urine phosphorus concentration of calculi groups of non-affected wethers was greater than the urine phosphorus concentra-

TABLE 4. PERCENTAGE OF MINERAL EXCRETION VIA THE URINE (% U)[a] IN EXPERIMENT II

Group Treatment	5 Basal	6 Calcium citrate
No. of wethers	5	9
Daily urine volume, ml.	789	531[b]
Calcium, % U	0.6	0.5
Magnesium, % U	33.3	32.8
Phosphorus, % U	7.0	0.7[c]
Potassium, % U	91.2	96.5
Sodium, % U	64.0	86.2[b]

[a] $\% U = \frac{\text{mg. mineral voided in urine}}{\text{mg. mineral voided in urine and feces}} \times 100.$
[b] Significantly different ($P<.05$) from group 5.
[c] Significantly different ($P<.01$), from group 5.

TABLE 5. MINERAL BALANCE OF WETHERS AS INFLUENCED BY DIET[a]

Mineral	Calcium		Magnesium		Phosphorus		Sodium		Potassium	
Group	5[b]	6[c]	5	6	5	6	5	6	5	6
Treatment	Basal	Calcium citrate	Basal	Calcium citrate	Basal	Calcium citrate	Basal	Calcium citrate	Basal	Calcium citrate
Intake	3.269	8.000	2.025	1.934	2.833	2.706	2.996	2.996	10.351	10.351
Feces	2.397	7.608	1.378	1.519	1.880	2.300	0.545	0.238	0.700	0.276
Urine	0.014	0.035	0.667	0.778	0.145	0.016	0.996	1.531[d]	7.279	7.777
Total excretion	2.411	7.643	2.045	2.297	2.025	2.316	1.541	1.769	7.979	8.053
Balance	+0.858[e]	+0.357	−0.020[e]	−0.363	+0.808[e]	+0.390	+1.455	+1.227	+2.372	+2.298

[a] Figures are average grams/day/wether. There were nine wethers per group during a 5-day trial.
[b] Group 5 is basal plus 0.5% sodium chloride.
[c] Group 6 is basal plus 2.95% calcium citrate plus 0.5% sodium chloride.
[d] Significantly different ($P<.05$) from group 5.
[e] Significantly different ($P<.05$) from group 6.

tion of calculi sheep. Phosphaturia is characteristic in all calculogenic diets in this experiment. Emerick *et al.* (1959) correlated elevated plasma and urine phosphorus with the development of urinary calculi.

Crookshank and Robbins (1962) reported that lambs developing calculi excreted urine of less magnesium concentration and more phosphorus concentration than do normal lambs. The current study (table 3) support this observation but the metabolism study shows further that the percent of total magnesium excreted via the urine is equal in calculogenic and non-calculogenic wethers while the percent of total phosphorus excreted via the urine is also increased in lambs developing calculi (table 4). A significant difference ($P<.05$) in urinary magnesium exists between the wethers on the calculi provoking vitamin D_3 supplemented diet and the wethers on the non-calculogenic calcium citrate diet (table 3). There is a significant difference ($P<.05$) in magnesium balance between the lambs on the basal calculi provoking diet (group 5, table 5) and lambs on the calcium citrate supplemented diet (group 6, table 5). Although the amount of urinary magnesium/day/wether (table 5) in the calcium citrate supplemented group (group 6) was higher than in the basal calculogenic group (group 5) the percent of magnesium voided via the urine is practically the same (table 4).

The non-calculi sheep (group 6, table 5) retain less phosphorus and magnesium and also excrete more phosphorus and magnesium via the feces than do the sheep with calculi (group 5). Since the calculi produced in this experiment consisted mainly of magnesium phosphate, the ability of the calcium citrate wethers to retain less phosphorus and magnesium and to excrete more of these two elements via the feces would appear to be an important factor in the prevention and reduction of calculi. As shown in table 5, the calcium citrate group also retained less sodium and excreted significantly more sodium ($P<.05$) via the urine.

Summary

The effects of some dietary factors on calcium, magnesium, phosphorus, sodium and potassium metabolism (as reflected in the urine and serum values and in metabolic balance studies) are compared with the incidence of urinary calculi in wethers.

In two experiments a total of 142 sheep were divided into seven different lots and were fed the basic calculogenic diet of Packett and Hauschild (1964), plus supplements consisting of sodium citrate, calcium citrate, vitamin D_3, or calcium carbonate.

A high incidence of calculi was observed in wethers receiving the basal diet and all supplemented diets except those supplemented with calcium citrate. The highest incidence of calculi (80%) occurred in the wethers supplemented with vitamin D_3; the lowest incidence (0–4%) in the wethers supplemented with calcium citrate.

The serum and urine of wethers on calculi provoking diets were characterized by high serum magnesium and phosphorus, high concentrations of urinary magnesium and phosphorus, and low serum calcium. Metabolism studies revealed that non-caluli wethers excreted more phosphorus and magnesium via the feces, excreted less magnesium and phosphorus via the urine, retained less magnesium and phosphorus and had lower serum magnesium and serum phosphorus levels.

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BY DIET[a]

	Potassium	
	5	6
um ate	Basal	Calcium citrate
996	10.351	10.351
238	0.700	0.276
531[d]	7.279	7.777
769	7.979	8.053
227	+2.372	+2.298

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Further Research on Decalcifiers and Convulsivants

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Siena Chapter - Session of July 20, 1953

Continuing the studies, the theoretical premises and the technique of which have been reported before (1), we wanted to see if the response to the "decalcifying" reagent differed, and to what extent, according to the "seat of action" of the convulsivants.

For this purpose there were injected first oxalate or citrate in nearly equivalent doses (g.eq./kg 0.0010) and at constant rates, and later, after different times from the end of the injection: caffeine, codeine, pyramidone, picrotoxin, phenol; drugs whose "seat of action" is known to be different.

We had determined the minimal effective doses of these drugs on the rabbit, the animal on which we were experimenting. In all cases the injections were made into the marginal vein of the ear.

We compile in the table, for each convulsivant, the minimal effective dose in g/kg and the minimal effective dose after injection of oxalate or citrate, including also strychnine, as in the previous reports (1, 2).

Drug	Min.effective dose g/kg 10^5	M.E.D. after oxalate	M.E.D. after citrate
Strychnine	13	13	15
Caffeine	8500-10100	7400-7800	7600-7800
Codeine	3400	2300-2700	3000
Pyramidone	7700	10000	10900
Picrotoxin	110	110	110
Phenol	1800	2100	2100

If instead of considering the minimal convulsivant dose we consider those which can be so in 25 or 50% of the cases, we note modifications in

the same sense after oxalate and after citrate. We can conclude, therefore, that oxalate, as it modifies the response of strychnine by increasing its action, modifies the response of caffeine and of codeine in the same sense. The same thing may be concluded for the citrate, although, as results from the doses reported, the increase of action of the caffeine and of the codeine is inconstant. Note that here drugs are involved which act chiefly by increasing the reflex excitability, even though in the case of codeine also a bulbar component occurs.

For the drugs, instead, with which the increase of reflex excitability is either absent or very small, and whose "seat of action" is in the spinal motor mechanisms (phenol) or especially in the bulbus and in the higher formations (pyramidone and picrotoxin), the action of the "decalcifier" is either almost zero or decreases the action of the convulsivant, as is shown by the fact that to obtain the convulsions it is necessary here to inject higher doses.

If we were to represent the phenomena in a schematic and elementary manner, we might expect that, as the increase of the active circulating calcium causes the action of the convulsivants to decrease more or less, its decrease causes this action to increase constantly. Indeed, the decrease in one case could correspond quantitatively to the increase in the other case.

For example, strychnine and pyramidone are subject to the antagonistic action of the calcium (3); we might expect, therefore, that by reducing the calcium with the "decalcifiers," the action of the two convulsivants would increase. In reality only strychnine confirms the prediction, while the action of the pyramidone decreases with the "decalcifiers." Probably, the more numerous the factors of the convulsivant action are, the more complicated becomes the interference with the "decalcifiers" on the nervous system.

Some considerations can make us aware of these different results. It must be easier to reduce simultaneously the excitability of various sectors of the C.N.S. by injecting calcium than to increase it by subtracting calcium with a "decalcifier." This may be argued from the fact that,

expecially for oxalate, always a certain time from the end of the injection is necessary before the conditions are established for an increase of the nervous excitability. On the other hand, there are drugs, like strychnine, whose action is, so to speak, simpler and sufficiently limited to certain nervous elements, and there are others, like pyramidone and picrotoxin, which no doubt act simultaneously on the greater part of the cerebrospinal axis. And when a "decalcification" is exerted simultaneously on various points of the C.N.S. which are connected in order to rank in the whole animal, a "decalcification" does not necessarily suggest an increase in activity of these convulsivants.

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(1) I. Tadei, this journal, 1952, vol. 28, 1340

(2) Same author, ibidem, 1953, 29, 575.

(3) L. Scremin, Rend. R. Accad. Naz. Lincei, Classe Sc. fis. matem.e. nat., 1st sem. 1928, 7, Series 6, No. 12, 1056.

(Translated by Carl Demrick Associates,Inc/LH/t)

e di μ 150 compresa la capsula. I glomeruli osservati nel tessuto interstiziale tra le papille, hanno un diametro medio di μ 162,5 esclusa capsula e 186,5 con la capsula, con massimo di μ 211, 5 resp. μ 236 ed un minimo di μ 87,5 e μ 112,5. L'area triangolare comprendente glomerulo e tubuli ha una superficie di mm^2 177 ca. Inoltre mi è apparso degno di nota il fatto di aver notato allo stesso livello di sezione due glomeruli, uno dei quali è quasi addossato alla parete di un'arteria, e l'altro completamente addossato alla parete della vena parallela ed adiacente all'a. stessa, nel punto diametralmente opposto a quello a ridosso dell'arteria. Entrambo i glomeruli hanno il loro apparato tubulare circostante e, naturalmente, distinto: l'apparato dal lato della vena è più evidente, forse per lo stadio funzionale al momento dell'osservazione, che non quello a ridosso dell'arteria.

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ULTERIORI RICERCHE SUI DECALCIFICANTI E CONVULSIVANTI.

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Sezione di Siena — Seduta del 20 luglio 1953.

Continuando nelle ricerche, sulle premesse teoriche e la tecnica delle quali è stato precedentemente riferito (1), abbiamo voluto vedere se la risposta al rettivo «decalcificante» differisse ed in quale misura, a seconda della «sede di azione» dei convulsivanti.

A questo scopo si iniettarono dapprima ossalato o citrato in dosi pressochè equivalenti (g.eq./kg 0,0010) ed a velocità costanti e, dopo, a tempi diversi dalla fine dell'iniezione: caffeina, codeina, piramidone, picrotossina, fenolo: farmaci la cui «sede di azione» è, come è noto, diversa.

Di questi farmaci avevamo determinato le dosi minime attive sul coniglio, animale sul quale si sperimentava. In tutti i casi le iniezioni furono fatte nella vena marginale dell'orecchio.

Raccogliamo nella tabella, per ogni convulsivante, la dose minima attiva in g/kg e la dose minima attiva dopo l'iniezione di ossalato o di citrato, includendo anche la stricnina, di cui alle note precedenti (1, 2).

Farmaco	Dose minima attiva g/Kg 10^5	d. m. a. dopo l'ossalato	d. m. a. dopo il citrato
Stricnina	18	13	15
Caffeina	8500 - 10100	7400 - 7800	7600 - 7800
Codeina	3400	2300 - 2700	3000
Piramidone	7700	10000	10900
Picrotossina	110	110	110
Fenolo	1800	2100	2100

Se invece di considerare la dose *minima convulsivante*, consideriamo quelle che possono esserlo nel 25 o 50% dei casi, notiamo dopo l'ossalato ed il citrato modificazioni nello stesso senso. Possiamo dunque concludere che l'ossalato, come modifica la risposta della stricnina *aumentandone* l'azione, modifica nello stesso senso la risposta della caffeina e della codeina. La stessa cosa si può concludere per il citrato, sebbene, come risulta dalle dosi riportate, l'aumento di azione della caffeina e della codeina sia incostante. Si osservi che trattasi qui di farmaci che agiscono prevalentemente aumentando l'eccitabilità reflessa, per quanto nel caso della codeina si abbia anche una componente bulbare.

Per i farmaci, invece, con i quali l'aumento di eccitabilità reflessa o manca o è molto piccolo, e la cui «sede di azione» è nei meccanismi motori spinali (fenolo) o soprattutto nel bulbo e nelle formazioni più alte (piramidone e picrotossina), l'azione del «decalcificante» o è *pressochè nulla* o *diminuisce l'azione del convulsivante*, come dimostra il fatto che ad ottenere le convulsioni, è necessario qui iniettare dosi più alte.

Se ci rappresentassimo i fenomeni in modo schematico ed elementare, potremmo attenderci che, come l'aumento del calcio attivo circolante fa diminuire più o meno l'azione dei convulsivanti, la sua diminizione facesse aumentare costantemente questa azione. Anzi, la diminuzione in un caso potrebbe corrispondere quantitativamente all'aumento nell'altro caso.

Ad esempio, stricnina e piramidone risentono dell'azione antagonistica del calcio (3); potremmo dunque attenderci che diminuendo il calcio con i «decalcificanti», l'azione dei due convulsivanti aumentasse. In realtà soltanto la stricnina conferma la previsione, invece l'azione del piramidone con i «decalcificanti», diminuisce. Probabilmente, più numerosi sono i fattori dell'azione convulsivante, e più complicata diventa l'interferenza con i «decalcificanti» sul sistema nervoso.

Alcune considerazioni possono renderci conto di questi diversi risultati. Deve essere più facile diminuire contemporaneamente l'eccitabilità di vari settori del S.N.C. con l'iniezione di calcio, che aumentarla sottraendo del calcio con un «decalcificante». Questo si può arguire dalla circostanza che, particolarmente per l'ossalato, è necessario sempre un certo tempo dalla fine dell'iniezione, perchè si stabiliscano le condizioni per un aumento dell'eccitabilità nervosa. D'altra parte vi sono farmaci come la stricnina la cui azione è, per così dire, più semplice e abbastanza limitata a certi elementi nervosi, e ce ne sono altri, come il piramidone e la picrotossina, che indubbiamente agiscono contemporaneamente sulla maggior parte dell'asse cerebro-spinale. E, quando nell'animale intero l'azione dei convulsivanti si esercita contemporaneamente su vari punti del S.N.C. che siano gerarchicamente collegati, una «decalcificazione» non può lasciar prevedere *necessariamente* un aumento di attività dei convulsivanti stessi.

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L'AZIONE ULCEROGE

(*Dall' Istituto*

Sezione

L'azione litica *in*
da R. Severi, il qual
luogo ove era stata i
L'enzima fu ino
In queste sedi sono
citoplasmatici, sulle
La lisi degli acidi ri
lula. Alla morte rap
consegue una perdita
rapidamente a guarig
I risultati ottenu
(Avezzù e Bonomi,
il liquido iniettato i
presenza in esso de
pancreas col motodo
sieme alla ribonucle
proteasi attive, di cu
ricco.
Tali enzimi avre
telio, o perlomeno p
In vero, enzimi
parazioni ribonucleas
la loro presenza spieg
ad essi sensibile. Tal
chè presentano una
parazioni di ribonuc
litica, se conveniente
quella nucleasica fin
nella genesi delle ul
enzimi proteolitici, e
nucleasi riscaldata.
Già Severi, nelle
constatato che l'azio
solo modicamente at
per 30 min. Questi r
crotizzante è fondam
Nelle ricerche su
Severi, che in quelle
adoperato un'enzima
Tale preparato c
centuale di protoasi;
sulla mucosa gastrica

From: Monathsschrift f. Kinderheilkunde, vol. 98, no. 7 p.311-314

On the effect of complex calcium nitrate on the organism

by W. Theopold and W. Hennig

The importance of calcium therapy is based on the fact that the calcium salts are an important component in cells and tissues and exceed quantitively all other salts in the organism. But a general view of the calcium metabolism in the body is difficult to obtain, as known. On the one hand, calcium is subject to a number of regulations which can not be de-limited from each other. An example are the parethyroid glands, the hypophysis, the adrenal glands, certain vitamins, and the interaction with the blood albumen and various ions. In addition, little is known about the intermediate circulation of calcium, because the calcium found in the excreta does not permit any conclusion upon a resorption that has taken place. It is thus not possible to distinguish whether it passed through the intestine unresorbed or whether it was subject to resorption and excretion during its passage through the intestine. The portion deposited in the skeleton as bone earth can thus not be determined quantitatively. These difficult conditions have led to vivid discussions about the effectiveness of orally applied calcium. While many researchers, like Steudel (1) recently, tend to believe that a calcium resorption from the intestine does not take place at all with this application, others are of the opposite view on the basis of their experiments.

This also explains why the need for calcium can not be exactly calculated, but only roughly estimated. We have to assume that the growing organism of the child needs larger quantities than that of grownups. If we consider that the daily requirement determined by Rubner (2) with 1.22 g, by v. Norden and Salomon (3) with 2.0 g, is valid, a normal diet should cover this requirement. An oversupply of calcium is not resorbed by the body, but a deficiency can soon lead to severe damages in the growing organism. But in grownups too, a prolonged inadequate supply leads to diseases, as the late rickets of the postwar years have proved.

There are numerous studies about the resorption of calcium. According to Aristoswky (4), it takes place substantially in the duodenum and jejunum, hence in sections of the intestines in which a slight acid to neutral reaction predominates. According to observations by Hesse (5) it is irrelevant in what form the calcium is offered, the solubility of the individual salts plays no role in the resorption. Important, however, is the reaction of the contents of the intestines; in an acid medium the resorption is improved, an an alkaline medium it is impaired. The solubility laws will thus hardly apply in the intestine as they apply for strictly hydrous solutions. These facts led Klinke (6) to the idea that calcium would be resorbed in the form of a lime soap-bile acid complex.

When Rominger (7) found the antirachitic effectiveness of sodium citrate again verified and proved it for the first time in human diseases, and when Glanzmann (8) reported on that of citric acid, both authors assumed that the calcium of the diet is combined to a complex by the supplied citrate-ions so that they are not precipitated by counter-ions and are easier resorbed. A complex bond between calcium and citric acid had already been found by Hastings (8) in tert. calcium citrate. On the basis of these investigations we asked ourselved what effect such a complex salt has in the organism if it is applied directly orally.

We prepared solutions in which we could demonstrate the complex bond between calcium and citric acid by means of conductivity titrations and of potentiometric measurements with the glass electrode (10). In this complex bond the calcium is no longer present as a cation, but it is a part of the anion. In the treatment of rachitic children it was found, however, that the oral application of such a calcium-citrate complex in a molar ratio of 1:4 leads to a drop of the serum calcium, sometimes to values down to 5 mg%. Manifest spasmophilia was not observed. Severe symptoms of hyperexcitability were thus prevented by the acidotic action of the citric acid and by a slight drop of the ionized portion in the serum calcium. Simultaneously a

decomposition of bone substance took place; in the radiographic representation the insufficient calcification on the epiphyses showed further deterioration. These findings can be interpreted as follows: When the calcium citrate complex passes over into the blood, the calcium is used for buffering to such an extent that the body must mobilize calcium substance from the tissues in order to keep the serum calcium level constant. With continued supply, however, it drops, despite this attempted compensation. Such a bond between calcium and citric acid has thus an adverse effect on the organism. But since Rominger and Glanzmann had achieved cures when the used sodium citrate instead of citric acid, their views on the formation of such a calcium-citrate complex in the form developed by them can not be correct. With the large doses of sodium citrate, as they had to be used by Rominger, it seems that the acidotic metabolism found in rickets has changed into a alkolotic metabolism. This alaklization probably plays an important role in the cure. We achieved cures in some cases with the small doses of citric acid indicated by Glanzmann. But this was in children which showed isolated cranial softening, which we can no longer consider today positively rachitic. In cases of definite rickets we saw first an improvement, but then an arrest was observed so that vitamin D had to be given. Winkler (11) and recently Sinios (12) were unsuccessful with the use of citric acid, the latter observed occasionally spasmophilia. We could show that the complex calcium citrate has a favorable effect only with the addition of an optimum phosphate-concentration. (13). Then a greater calcium deposit was found on the rachitic epiphyses with a gradual rise of the calcium level in the blood (13,14).

We were interested in the effect of such a complex compound on the organism. We investigated first whether oral doses would influence the calcium content of the blood, and for comparison we also administered other calcium salts. It seemed to us important to dose in the range of physiological calcium amounts, because disproportionally high doses had been used in previous tests. Thus Min-Sen Li (15) observed an increase of the calcium level

when he fed trialkol in a single or daily dose of 15 g for several days to babies and infants. Ehrenberg (16) used 3-4 g calcium per dose and Oxenius (15) considered a dose of 20 g per day particularly effective. In our investigations we gave a single dose which contained 200 mg calcium and calculated than the dose of the calcium salts studied for comparison.

Fig. 1 Serum calcium values. At the arrow the amount of salt containing 0.2 g Ca was given.

Fig. 2 Ca-, P- and citric acid values in the blood, At the arrow 0.2 g Ca contained in a complex compound were given.

Fig. 3 Ca-,P- and citric acid values in the blood. At the arrow 4.0 g citric acid were given.

Normally the calcium level of grownups is kept constant in the course of the day. Even after meals there are no conspicuous fluctuations. But in order to exclude as far as possible disturbing factors, the test persons, almost exclusively healthy young men, started the tests on an empty stomach and took no food whatsoever during the entire test period. They were kept in rest position. After a first blood sampling, the respective substance was administered perorally in solution, then blood was taken again in certain time intervals. The calcium was determined according to the Kramer-Tisdall method, in each case double determinations were made.

The results of the tests are represented in the form of mean value curves from 6 tests each. We see that peroral doses of calcium chloride, tert. calcium-citrate, sodium-calcium lactate have little effect on the calcium level, while calcium gluconate has a varying effect. A rapid and lasting increase of the serum calcium is achieved after applications of the complex calcium citrate with phosphate. (fig.1). If we wanted to conclude that this increase is due to the resportion of the supplied calcium, this would have to take place from the duodenum or jejunum, not later. If we apply

a second dose (fig.2), after the effect of the first has worn out, we find again a considerable increase of the serum calcium, which drops faster, however, than the first time, sometimes below the starting point. If we want to obtain a favorable effect, we have to prevent acidosis by applying the single doses in corresponding time intervals. This rise was also found in comparative individual tests in babies.

With peroral application of pure citric acid (fig.3) we find likewise an increase of the serum calcium, but it is not so pronounced as with applications of the complex salt, and it drops again rapidly. Simultaneous determinations of the serum-citric acid (Shhorr-Traussky method) show a rise of the citrate level, which is even more pronounced with applications of the complex.

These results alone justify by no means the conclusion that the complex must have an adverse effect. Particularly from the temporary greater rise of the serum citric acid it could be assumed that calcium salts are mobilized for their buffering. This limitation seems to be important to us, since many authors as well as Braun (18) is a previously published monography concluded from the increase of the serum calcium simply upon a resorption. From these results alone it can not be determined whether the supplied calcium was resorbed or whether the increase of the serum calcium level is due to the calcium possibly mobilized from the tissues. Such a therapy would lead in the last analysis to increased elimination of calcium and would withdraw so much calcium from the body that it would lead to osteoporosis.

A better survey is obtained by demonstrating that the calcium is retained. In order to determine a possible retention after doses of the complex calcium salt, we made feeding tests on rats. We fed young growing rats with different diets and determined than, in addition to the ash content, the amount of calcium and phosphate in the bones. The first group was fed with kitchen leftovers, the second received a modified rachitogenic diet according to Steinbock-Black with a Ca/P quotient of 5:1. In a third group the complex

was added to this diet so that a quotient of 4.3:1 resulted, which was thus not always in the positively rachitic range. When the animals were sacrificed, the average weight in the various groups was about 70 g. The results are compiled in table 1.

Table 1

ash content			normal diet		rach. diet		rach.diet + complex	
norm. %	rach. %	complex %	Ca mg%	P mg%	Ca mg%	P mg%	Ca mg%	P mg%
41.3	35.7	55.7	14.3	7.3	13.0	6.0	21.2	10.7
43.2	20.4	51.4	15.3	7.8	7.5	3.1	19.2	8.4
42.4	33.4	54.0	15.2	8.1	10.9	6.1	18.2	9.3
46.6	33.1	55.7	17.3	8.7	11.5	4.7	21.5	9.9
42.3	23.6	51.4	15.1	8.3	8.5	4.3	18.8	10.0
44.3	29.0	58.0	15.3	7.5	10.0	5.9	23.1	10.2
43.9	28.5	56.0	15.8	7.2	12.3	4.3	22.7	9.5
40.0	30.6	-	13.5	7.7	10.1	4.7	-	-
43.0	28.0	54.6	15.2	7.8	10.5	4.9	20.9	9.7

The addition of the complex calcium citrate with phosphate to a definitely rachitogenic deficiency diet results thus in the young growing rat in the deposit of calcium and phosphate in the bones. The mineral content is exceeded in the animals on a normal diet. Furthermore the occurrence of rickets can be prevented: while all animals of the 2nd group suffered from a severe case of rickets, all animals of the third group were spared. In the histological examination of the kidneys, no calcium deposit was found in any of the animals.

We also drew up calcium balances in two healthy children. We proceeded according to the method indicated by Kueng (19) by carrying out the tests in 3 periods of 5 days each. During a preliminary period we got an idea of the intake and excretion, during the second period the preparation was given additionally, and in the third period any subsequently appearing excessive

excretion of the mineral was determined. The investigation comprised thus 15 days during this time the children were kept in bed. They received during this time a lactovegetable diet which was analyzed exactly for its content of calcium and phosphate. In putting together this diet, care was taken that the daily requirement of calcium as demanded by Rubner, was met. In the analysis of the foods we found:

Table 2

food	mg% Ca	mg% P
boiled potatoes	4.8	24.4
peas (canned) with water	24.2	35.1
carrots (canned)	26.4	17.4
baked plums (without pits)	179.0	150.0
raisins	141.0	144.0
zwieback	225.0	272.0
flour	20.0	121.0
corn starch	25.1	39.7
powdered milk	990.0	750.0

The result of the tests is shown in fig. 4 and 4a. In both cases we find retention of the minerals Ca and P. In the former case an initially negative balance changes to positive after the medication was given. In the second investigation too, we find a clearly improved balance after the supply of the complex. The retention, which is particularly conspicuous here, is weakened in the post period by increased excretion. By the application of the complex the excreted amounts of Ca and P are reduced. According to investigations by Steggerida and Mitchell (20) such an effect is not obtained with pure citric acid; they found a considerably increased excretion of calcium in the feces after administration of this substance. Pure lemon juice can not influence either the mineral metabolism to this extent. Animal experiment by Mouriquand (24) showed that the addition of lemon juice to a Ca-deficiency diet causes the appearance of osteophytes.

If we summarize the effect of calcium-citric acid complex on the organism, we find:

1. Pure calcium citric complex compounds have an adverse effect by lowering the serum calcium level and by an increased decomposition of bone substance.

Fig. 4 Balance, child, Ernest B. 10 years old.

Fig. 5 Balance, child Karin D. 10 years old.

2. This adverse effect is transformed into the opposite, namely an increase of the serum calcium level and buildup of bone substance, if an optimum amount of phosphate is added to the complex.

3. In this composition an oral dose which contains 200 mg calcium effects a lasting rise of the serum calcium, which was not observed with equal doses of some other calcium salts.

4. In the animal experiment, the feeding of such a complex led to a considerable enrichment of the bone with Ca and P, without any pathological calcium deposites in the body.

5. With oral applications, calcium contained in a complex compound is retained after a corresponding addition of phosphate, salt can be seen from the metabolism balances.

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(Translated by Carl Demrick Associates,Inc/IE/t)

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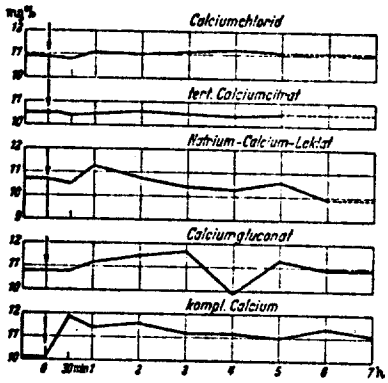


Fig. 1.

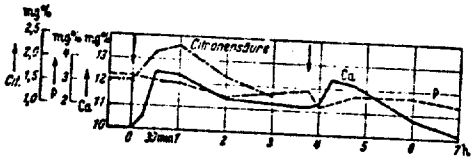
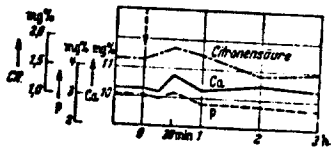


Fig. 2.



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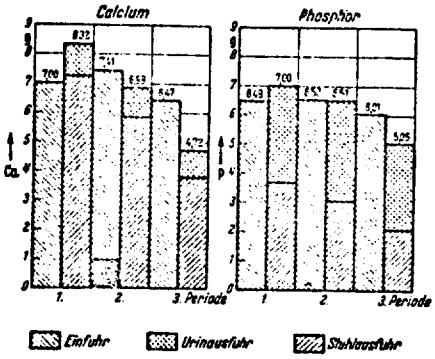

Fig. 4.

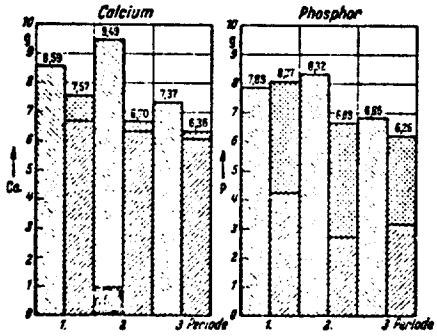

Fig. 5.

Aus der Kinderklinik (Komm. Direktor: Prof. Dr. LINNEWEH) und dem Pharmakologischen Institut (Komm. Direktor: Prof. Dr. HEIM) der Universität Marburg/Lahn.

Über die Wirkung von komplexem Calciumcitrat auf den Organismus.

Von W. THEOPOLD und W. HENNIG.

(*Eingegangen am 28. März 1950.*)

Die Wichtigkeit einer Kalktherapie begründet sich schon durch die Tatsache, daß die Calciumsalze ein wichtiger Bestandteil in Zellen und Geweben sind und mengenmäßig alle anderen Salze im Organismus übertreffen. Ein Überblick über den Calciumstoffwechsel im Körper läßt sich aber bekanntlich schwer erlangen. Einmal unterliegt das Calcium einer Fülle von Regulationen, die sich gegeneinander nicht abgrenzen lassen, man denke an die Nebenschilddrüsen, die Hypophyse, die Nebennieren, gewisse Vitamine und die Wechselwirkung mit dem Bluteiweiß und verschiedenen Ionen. Außerdem kann man aber auch deshalb wenig über den intermediären Kreislauf des Calciums aussagen, weil das in den Exkreten gefundene Calcium keinen Rückschluß auf eine stattgehabte Resorption erlaubt. Man kann also nicht unterscheiden, ob es unresorbiert den Darm passierte oder einer Resorption und Exkretion bei der Darmpassage unterlag. So ist der im Skelet als Knochenerde abgelagerte Anteil mengenmäßig gar nicht faßbar. Diese schwer übersichtlichen Verhältnisse haben zu lebhaften Auseinandersetzungen über die Wirksamkeit des oral applizierten Calciums geführt. Während viele Untersucher, wie in letzter Zeit STEUDEL [1], zu der Ansicht neigen, daß eine Calciumresorption aus dem Darm bei dieser Anwendung überhaupt nicht stattfinde, vertreten andere auf Grund ihrer Experimente den gegenteiligen Standpunkt.

Daher erklärt es sich auch, daß sich der *Bedarf an Calcium* nicht genau berechnen, sondern nur überschlagsweise schätzen läßt. Man wird dem wachsenden Organismus des Kindes eine größere Menge zubilligen müssen als dem des Erwachsenen. Wenn man den von RUBNER [2] mit 1,22 g, von v. NOORDEN und SALOMON [3] mit 2,0 g festgesetzten Tagesbedarf als gültig betrachtet, so dürfte eine normale Ernährung diesen Bedarf decken. Ein Überangebot an Calcium wird vom Körper nicht resorbiert, dagegen kann ein Mangel beim wachsenden Organismus sehr bald zu schweren Schäden führen. Aber auch bei Erwachsenen ruft eine längere unzureichende Zufuhr Krankheiten hervor, wie die Hungerosteopathien der Nachkriegsjahre beweisen.

Über die *Resorption des Calciums* liegen zahlreiche Untersuchungen vor, nach ARISTOWSKY [4] vollzieht sie sich im wesentlichen in Duodenum und Jejunum, also in Darmabschnitten, in denen eine leicht saure bis neutrale Reaktion herrscht. Nach den Beobachtungen von HESSE [5] ist es gleichgültig, in welcher Form das Calcium angeboten wird, die Löslichkeit der einzelnen Salze spielt für die Resorption keine Rolle, auch nichtwasserlösliche Salze werden resorbiert. Wichtig ist dagegen die Reaktion des Darminhaltes; in saurem Milieu wird die Resorption verbessert, im alkalischen verschlechtert. Im Darm werden also schwerlich die Löslichkeitsgesetze Gültigkeit haben wie sie für rein wäßrige Lösungen zutreffen. Diese Tatsachen führten KLINKE [6] zu der Auffassung, daß das Calcium in Form eines Kalkseifen-Gallensäure-Komplexes aufgenommen würde.

Als ROMINGER [7] die *antirachitische Wirksamkeit des Natriumcitrates* erneut bestätigt fand und erstmalig bei der Erkrankung am Menschen bewies und GLANZMANN [8] über die der *Citronensäure* berichtete, nahmen beide Autoren an, daß das Calcium der Nahrung durch die zugeführten Citrationen komplex gebunden werde, damit einer Fällung durch Gegenionen entgehe und leichter zur Resorption gelange. Eine komplexe Bindung zwischen Calcium und Citronensäure war bereits von HASTINGS [9] im tertiären Calciumcitrat bewiesen worden. Im Anschluß an diese Untersuchungen haben wir uns die Frage vorgelegt, welche Wirkungen ein solches *Komplexsalz* im Organismus entfaltet, wenn man es *direkt oral* zuführt.

Wir stellten Lösungen her, in denen wir die komplexe Bindung des Calciums an die Citronensäure mit Hilfe von Leitfähigkeitstitrationen und von potentiometrischen Messungen mit der Glaselektrode nachweisen konnten [10]. In dieser komplexen Bindung liegt das Calcium nicht mehr als Kation vor, sondern es ist Bestandteil des Anions. Bei der Behandlung rachitischer Kinder zeigte sich aber, daß die orale Zufuhr eines solchen Calcium-Citrat-Komplexes im Molverhältnis von 1:4 zu einem Absinken des Blutcalciums, manchmal bis auf Werte bis zu 5 mg-%, führte. Manifeste Spasmophilie sahen wir dabei nicht auftreten. Schwere Erscheinungen der Übererregbarkeit wurden also durch die acidotische Wirkung der Citronensäure und durch ein geringes Absinken des ionisierten Anteils am Blutcalcium verhindert. Gleichzeitig fand ein Abbau von Knochensubstanz statt, bei röntgenologischer Darstellung erfuhr die mangelhafte Verkalkung an den Epiphysen eine weitere Verschlechterung. Diese Befunde lassen sich so deuten: beim Übertritt des Calcium-Citrat-Komplexes in das Blut wird zu seiner Pufferung in so großem Maße das Calcium-Ion herangezogen, daß der Körper Kalksubstanz aus den Geweben mobilisieren muß, um den Blutkalkspiegel konstant zu halten. Bei fortgesetzter Zufuhr sinkt dieser aber trotz dieses Kompensationsversuches ab. Eine solche Verbindung zwischen Calcium und Citronensäure ist also von ungünstiger Wirkung auf den Organismus. Da aber ROMINGER und GLANZMANN Heilungen erreichten, wenn sie Natriumcitrat bzw. Citronensäure gaben, können ihre Ansichten über die Bildung eines solchen Calciums-Citrat-Komplexes in der von ihnen entwickelten Form nicht zutreffend sein. Vielmehr dürfte bei den großen Dosen von Natriumcitrat, wie sie ROMINGER anwenden mußte, die bei der Rachitis vorliegende acidotische Stoffwechsellage in eine alkalotische übergehen. Dieser Alkalisierung dürfte ein wichtiger Effekt bei der Heilung zukommen. Mit den von GLANZMANN angegebenen geringen Dosen von Citronensäure haben wir in einzelnen Fällen Heilungen erreicht. Es handelte sich aber dabei um Kinder, die lediglich eine *isolierte Schädelerweichung* aufwiesen und die wir heute *nicht mehr als sicher rachitisch* ansehen können. In Fällen eindeutiger Rachitis sahen wir zwar zunächst eine Besserung, dann trat aber ein Stillstand

ein, so daß die Anwendung des Vitamins D erforderlich wurde. WINKLER [11] und neuerdings SINIOS [12] hatten bei der Verwendung von Citronensäure keine Erfolge, letzterer sah gelegentlich eine Spasmophilie auftreten. Wir konnten nachweisen, daß das *komplexe Calciumcitrat erst bei Zusatz einer optimalen Phosphatkonzentration günstig* wirkt [13]. Nun fand sich eine stärkere Kalkeinlagerung an den rachitischen Epiphysen bei allmählichem Anstieg des Calciumspiegels im Blut [13, 14].

Uns interessierte nun die Wirkung einer solchen Komplexverbindung auf den Organismus. Wir untersuchten zunächst, ob perorale Gaben den Calciumgehalt des Blutes beeinflussen würden, und gaben aus

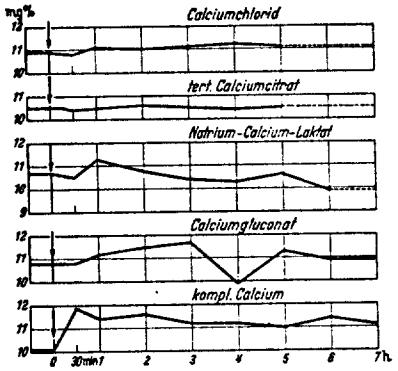

Abb. 1. Blutcalcium-Werte. Bei ↓ wurde jeweils die 0,2 g Ca enthaltende Salzmenge gegeben.

Vergleichsgründen auch andere Calciumsalze. Es erschien uns wichtig, im Bereich physiologischer Calciummengen zu dosieren. Bei bisherigen Untersuchungen wurden nämlich vielfach unverhältnismäßig hohe Dosen verwendet. So sah MIN-SEN LI [15] eine Erhöhung des Calciumspiegels auftreten, wenn er an Säuglinge und Kleinkinder mehrere Tage lang Trikalkol in einer Einzel- bzw. Tagesdosis von 15 g verfütterte. EHRENBERG [16] verwandte 3—4 g Calcium pro Dosis und OXENIUS [15] sah eine Dosis von 20 g pro die als besonders wirksam an. Bei unseren Untersuchungen gaben wir eine Einzeldosis, die 200 mg Calcium enthielt, und berechneten danach die Dosis der zum Vergleich untersuchten Kalksalze.

Normalerweise wird der Kalkspiegel des Erwachsenen im Laufe des Tages weitgehend konstant gehalten. Auch nach den Mahlzeiten treten keine auffallenden Schwankungen auf. Um aber störende Faktoren möglichst auszuschließen, traten die Versuchspersonen, fast ausschließlich *gesunde junge Männer*, nüchtern in den Versuch und nahmen während der Versuchsdauer keinerlei Nahrung zu sich. Sie wurden in Ruhelage gehalten. Nach einer ersten Blutentnahme wurde die betreffende Substanz in Lösung peroral gegeben, dann wurde in bestimmten Zeitabständen wieder Blut entnommen. Die Calcium-Bestimmung erfolgte nach der Methode von KRAMER-TISDALL, in jedem Fall wurden Doppelbestimmungen durchgeführt.

Die Untersuchungsergebnisse haben wir in Form von Mittelwertskurven aus je 6 Versuchen dargestellt. Es zeigt sich, daß perorale Gaben von Calciumchlorid, tertiärem Calciumcitrat, Natrium-Calciumlactat den Calciumspiegel nur wenig, von Calciumglukonat wechselnd beeinflussen. Eine *rasche und anhaltende Erhöhung des Serumcalciums* wird aber nach Gaben des komplexen Calciumcitrats mit Phosphat erreicht (Abb. 1). Wollte man daraus schließen, daß diese Erhöhung durch die Resorption des zugeführten Calciums bedingt sei, so müßte diese vom Duodenum oder Jejunum aus, nicht später, erfolgt sein. Gibt man, nachdem die Wirkung der ersten abgeklungen ist,

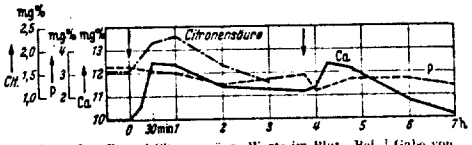

Abb. 2. Ca-, P- und Citronensäure-Werte im Blut. Bei ↓ Gabe von 0,2 g komplex gebundenem Ca.

eine zweite Dosis (Abb. 2), so erfolgt alsbald wieder ein erheblicher Anstieg des Serumkalkes, der aber schneller als das erstemal abfällt und unter den Ausgangswert sinken kann. Wenn man eine günstige Wirkung erhalten will, muß man eine Acidosis dadurch zu verhindern suchen, daß man die Einzeldosen in entsprechenden Zeitabständen appliziert. Bei vergleichenden Einzeluntersuchungen *beim Säugling* konnten wir diesen *Anstieg ebenfalls* nachweisen.

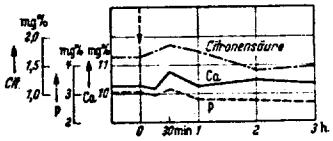

Abb. 3. Ca-, P- und Citronensäure-Werte im Blut. Bei ↓ Gabe von 4,0 g Citronensäure.

Bei peroraler Zufuhr *reiner Citronensäure* (Abb. 3) zeigt sich ebenfalls eine Erhöhung des Serumkalkes, diese ist aber nicht so ausgeprägt wie bei Gaben des Komplexsalzes und klingt schnell wieder ab. Gleichzeitige Bestimmungen der Blut-Citronensäure (Methode nach SHORR und THAUSSKY) lassen einen Anstieg des Citratspiegels erkennen, der bei Gaben des Komplexes stärker ist.

Diese Ergebnisse allein berechtigen keineswegs zu der Schlußfolgerung, der Komplex müsse von günstiger Wirkung sein. Gerade aus dem vorübergehend stärkeren Anstieg der Blut-Citronensäure könnte man vermuten, daß zu ihrer Pufferung Kalksalze mobilisiert werden. Diese Einschränkung erscheint uns sehr wichtig, da bei vielen Autoren wie auch in einer vor kurzem erschienenen Monographie von BRAUN [18] aus der Erhöhung des Serumkalkes einfach auf eine Resorption geschlossen wird. Aus solchen Ergebnissen allein läßt sich nicht ermitteln, ob das zugeführte Calcium resorbiert wurde oder ob die Steigerung des Serumkalkspiegels auf das möglicherweise aus den Geweben mobilisierte Calcium zurückzuführen ist. Eine solche Therapie würde letzten Endes zu einer vermehrten Ausscheidung von Calcium führen und dem Körper so viel Kalk entziehen, daß es zu einer Osteoporose kommt.

Ein besserer Überblick läßt sich durch den Nachweis gewinnen, daß das *Calcium retiniert* wird. Um eine mögliche Retention nach Gaben des komplex gebundenen Calciums festzustellen, wurden *Fütterungsversuche an Ratten* vorgenommen. Wir ernährten junge

wachsende Tiere mit verschiedener Kost und bestimmten dann neben dem Aschegehalt die Menge des Calciums und des Phosphates im Knochen. Die erste Gruppe wurde mit Küchenabfällen gefüttert, die zweite erhielt eine modifizierte rachitogene Kost nach STEENBOCK-BLACK mit einem Ca:P-Quotienten von 5:1. Einer dritten Gruppe setzten wir zu dieser Diät den Komplex zu, so daß ein Quotient von 4,3:1 resultierte, der also noch immer im sicher rachitogenen Bereich lag. Bei der Tötung betrug das Durchschnittsgewicht der Tiere in den einzelnen Gruppen etwa 70 g. Die Ergebnisse finden sich in der Tabelle 1 zusammengestellt.

Tabelle 1.

Aschegehalt			Normalkost		Rach. Kost.		Rach. Kost + Kompl.	
Norm. %	Rach. %	Komplex %	Ca mg %	P mg %	Ca mg %	P mg %	Ca mg %	P mg %
41,3	35,7	55,7	14,3	7,3	13,0	6,0	21,2	10,7
43,2	20,4	51,4	15,3	7,8	7,5	3,1	19,2	8,4
42,4	33,4	54,0	15,2	8,1	10,9	6,1	18,2	9,3
46,6	33,1	55,7	17,3	8,7	11,5	4,7	21,5	9,9
42,3	23,6	51,4	15,1	8,3	8,5	4,3	18,8	10,0
44,3	29,0	58,0	15,3	7,5	10,0	5,9	23,1	10,2
43,9	28,5	56,0	15,8	7,2	12,3	4,3	22,7	9,5
40,0	30,6	—	13,5	7,7	10,1	4,7	—	—
43,0	28,0	54,6	15,2	7,8	10,5	4,9	20,9	9,7

Die Zufütterung des komplexen Calciumcitrates mit Phosphat zu einer ausgesprochen rachitiserzeugenden Mangelkost bewirkt also bei der jungen wachsenden Ratte eine Einlagerung von Calcium und Phosphat im Knochen. Dabei wird der Mineralgehalt beim normal ernährten Tier übertroffen. Weiter läßt sich das Auftreten einer Rachitis verhüten: während alle Tiere der 2. Gruppe an einer schweren Rachitis erkrankten, blieben alle Tiere der 3. Gruppe verschont. Bei der histologischen Untersuchung der Nieren wurden bei keinem Tier Kalkablagerungen gefunden.

Ferner führten wir *bei 2 gesunden Kindern Calciumbilanzen* durch. Wir verfuhren dabei nach der von KÜNG [19] angegebenen Methode, indem wir die Untersuchung in 3 Perioden zu je 5 Tagen durchführten. Während einer Vorperiode wurde ein Überblick über Aufnahme und Ausscheidung gewonnen, in der 2. Periode wurde zusätzlich das Präparat gegeben, eine 3. Periode wurde angeschlossen, um eine eventuell nachträglich auftretende überschießende Ausscheidung der Mineralien zu erfassen. Die Untersuchung umfaßte also 15 Tage, während dieser Zeit wurden die Probanden bei Bettruhe gehalten. Die Kinder bekamen in dieser Zeit eine lactovegetabilische Kost, die genau auf ihren Gehalt an Calcium und Phosphat analysiert wurde. Es wurde bei der Zusammenstellung dieser Kost darauf geachtet, daß der von RUBNER geforderte Tagesbedarf an Calcium eingehalten wurde. Bei der Analyse der Nahrungsmittel fanden wir:

Tabelle 2.

Nahrungsmittel	mg-% Ca	mg-% P
Gekochte Salzkartoffeln	4,8	24,4
Erbsen (Konserven) mit Wasser	24,2	35,1
Möhren (Konserven)	26,4	17,4
Backpflaumen (ohne Steine)	179,0	150,0
Rosinen	141,0	144,0
Zwieback	225,0	272,0
Mehl	20,0	121,0
Mondamin	25,1	39,7
Milchpulver	990,0	750,0

Das Ergebnis der Untersuchungen findet sich in den Abb. 4 und 4a. In beiden Fällen ergibt sich eine *Retention der Mineralien Ca und P*. Im ersteren Fall schlägt eine anfangs negative Bilanz ins Positive um, nachdem das Medikament zugeführt wurde. Auch bei der zweiten Untersuchung zeigt sich eine deutlich ver-

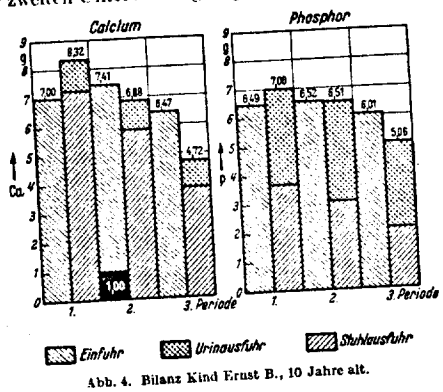

Abb. 4. Bilanz Kind Ernst B., 10 Jahre alt.

besserte Bilanz nach Zufuhr des Komplexes. Die Retention, die hier besonders auffallend ist, wird auch in der Nachperiode nicht durch eine gesteigerte Ausscheidung abgeschwächt. Unter Gaben des Komplexes werden die ausgeschiedenen Mengen an Ca und P vermindert. Nach den Untersuchungen von STEGGERDA und MITCHELL [20] trifft eine solche Wirkung für die reine Citronensäure nicht zu, sie fanden in Stoffwechselbilanzen nach Gaben dieser Substanz eine erheblich verstärkte Ausscheidung von Calcium im Stuhl. Auch reiner Citronensaft vermag den Mineralstoffwechsel nicht in diesem Maße zu beeinflussen. Tierexperimentelle Untersuchungen von MOURIQUAND [24] ergaben, daß eine Zufütterung von Citronensaft zu einer Ca-Mangelkost das Auftreten von Osteophyten bewirkt.

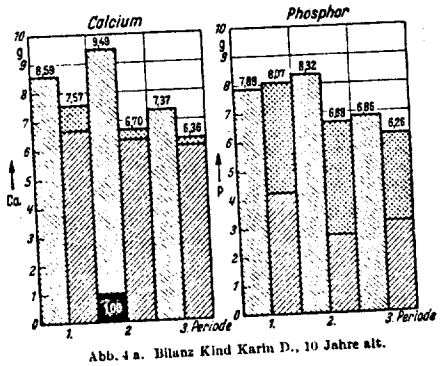

Abb. 4 a. Bilanz Kind Karin D., 10 Jahre alt.

Fassen wir die Wirkung des Calciums-Citronensäure-Komplexes auf den Organismus *zusammen*, so stellen wir fest:

1. Reine Calcium-Citronensäure-Komplexverbindungen wirken durch Senkung des Blutkalkspiegels und vermehrten Abbau von Knochensubstanz nachteilig.

2. Diese nachteilige Wirkung wandelt sich in das Gegenteil um, nämlich Hebung des Calciumspiegels und Anbau von Knochensubstanz, wenn man dem Komplex eine optimale Menge an Phosphat zusetzt.

3. In dieser Zusammensetzung bewirkt eine orale Gabe, die 200 mg Calcium enthält, einen anhaltenden Anstieg des Serumcalciums, wie er bei gleichgroßen Gaben einiger anderer Calciumsalze nicht beobachtet wurde.

4. Im Tierexperiment führt die Verfütterung eines solchen Komplexes zu einer erheblichen Anreicherung des Knochens an Ca und P, ohne daß es zu pathologischen Kalkablagerungen im Körper käme.

5. Oral verabfolgt, wird komplex gebundenes Calcium bei entsprechendem Phosphatzusatz retiniert, wie sich aus Stoffwechselbilanzen nachweisen läßt.

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Gibt es eine „Fibrinasthenie" als Ursache von Blutungsbereitschaft bei schwerer Lues connata?

Von HERBERT BURGSTEDT.

(*Eingegangen am 12. 4. 1950.*)

Als Material für den Faserstoff des jede Gefäß- und Gewebsverletzung verschließenden Thrombus hat das Fibrinogen eine evidente Bedeutung bei der Blutstillung. Seine Bildung ist im retikuloendothelialen System anzunehmen und somit nicht an ein einzelnes Organ gebunden. Eine Fibrinogenopenie kann bekanntlich angeboren oder, bei schweren Infektionen oder ausgedehnten Tumormetastasen, erworben auftreten. Sie führt zu den klinischen Zeichen einer hämorrhagischen Diathese, wenn ein — relativ tiefliegender — Schwellenwert im Blutspiegel unterschritten wird. Es ist theoretisch denkbar, daß auch eine *qualitativ* beeinträchtigte Fibrinogenbildung, die sich dann auf die Bildungszeit und die Festigkeit des Thrombus auswirken müßte, zu einer Blutungsneigung führt. Eine derartige Fibrinasthenie hat FANCONI 1941 erstmalig bei einem Säugling mit Lues connata angegeben und seitdem bei 4 weiteren Fällen gefunden.

Eine Blutungsneigung infolge oder bei angeborener Syphilis war vorher schon in seltenen Fällen (DELBECK, FINKELSTEIN, ERLICH u. a.) beschrieben worden, wobei sie einen besonders malignen, tödlichen Verlauf der Krankheit kennzeichnete, wie auch sämtliche von FANCONI erwähnten Kinder innerhalb kurzer Zeit starben. Bei der Deutung dieses Symptoms wurde von den Autoren eine besondere Manifestierung der luischen Infektion etwa in Form einer lokalisierten Gefäßerkrankung, wie sie die Bezeichnung „Lues congenita hämorrhagica" ausdrücken sollte, infolge des meist diffusen Charakters der Blutungen abgelehnt. Recht allgemein wird auf eine infektiös-toxische Schädigung des Organismus durch die „Spirochätensepsis" und eine im Einzelfall vorhandene, konstitutionelle Minderwertigkeit des Blutstillungsmechanismus hingewiesen. Diesen Erklärungen stellt FANCONI die für das Zustandekommen der hämorrhagischen Diathese alleinige oder wesentliche Bedeutung einer verminderten Festigkeit des Thrombus infolge Fibrinasthenie gegenüber.

Bei den von ihm erwähnten Fällen fand sich als zunächst auffallendstes Symptom eine hochgradige Labilität des Plasmas, die zu teils fetttröpfchenartigen, teils netzartigen Ausfällungen einer vom Verfasser bereits als Fibrinogen festgelegten Eiweißsubstanz im auf gewöhnliche Weise angefertigten Blutausstrichpräparat führte. Die WEIGERTsche Fibrinfärbung wurde von diesen Niederschlägen nicht angenommen, diese blieben im Gegenteil als Aussparungen im leicht angefärbten Plasma sichtbar. Der Fibrinogenspiegel war normal, nach der letzten Arbeit leicht erhöht. Im Gegensatz zu dieser leichten Fällbarkeit stand der Befund einer zum Teil erheblich verlängerten Gerinnungszeit, der — bei immer normaler Prothrombinzeit! — nur auf der Veränderung des Faserstoffes beruhen konnte. Diese zunächst widerspruchsvolle Tatsache wird von FANCONI in der Weise gedeutet, daß das qualitativ veränderte Fibrinogen zwar beschleunigt ausfällt, ohne jedoch richtige Fibrinfäden zu bilden, so daß es — verzögert — zur Bildung eines minderwertigen Gerinnsels und damit zur Ursache der hämorrhagischen Diathese kommt.

Entsprechend der Vermehrung grob-disperser labiler Eiweißfraktionen (bei der kataphoretischen Untersuchung war sowohl der γ-Globulin- als auch der Fibrinogenbereich relativ erhöht) war die Viscosität des Plasmas trotz verminderten Gesamteiweißgehaltes erhöht, die Takata-Ara-Reaktion positiv, das WELTMANNsche Koagulationsband verbreitert, sowie die Blutsenkungsreaktion beschleunigt.

Die klinischen Erscheinungen stimmten in allen Fällen weitgehend überein. Die Störung trat nach anfänglichem Gedeihen akut bis subakut im Laufe des zweiten Lebensmonates auf und führte mit progredientem Verfall, zum Teil unter den Zeichen einer Ernährungsstörung zum Tode. Es bestanden diskrete Zeichen einer hämorrhagischen Diathese, besonders Nasenbluten, und die Blutungszeit war leicht verlängert. Ödeme wurden vereinzelt, eine hochgradige

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Experimental Studies

Sodium and Potassium Citrate Salts for the Treatment of Digitalis Toxicity*

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It is paradoxic that after 178 years of clinical use it appears that the incidence of toxic effects from the administration of digitalis is increasing.[1-4] It has been variously estimated that between 6 and 20 per cent of patients receiving digitalis show some evidence of toxic sequelae.[1,5] This increased incidence of digitalis toxicity has been attributed to the use of saluretic drugs with the glycosides for the management of congestive heart failure, hypertension and edema.[4] The diuretic compounds cause a loss of potassium which makes the myocardium more sensitive to the action of digitalis.[4,6-9] This might be called digitalis sensitivity rather than toxicity, because in a patient on a previously tolerated dose of digitalis, severe disturbances of cardiac rhythm and conduction may develop when the myocardial intracellular potassium is reduced.

There are many toxic effects of digitalis such as nausea, vomiting, diarrhea, and disturbed vision; but they are of little significance compared to the cardiac disturbances of rhythm and conduction.[4] Two main types of arrhythmias may result: ectopic arrhythmias arising in any part of the atria, A-V node or ventricles, and conduction defects resulting in intraatrial block or various degrees of A-V block and intraventricular block. These arrhythmias are often lethal, the mortality rate rising to 65 per cent when supraventricular tachycardia with block occurs.[6]

Digitalis-induced arrhythmias may be treated by influencing the serum calcium-potassium ratio. This can be effected by the administration of potassium or by the administration of calcium-chelating agents. If the stores of myocardial potassium are depleted, they may be restored by administration of oral or intravenous potassium. The intravenous administration of potassium must be considered a dangerous procedure because it also causes conduction disturbances similar to those of digitalis.[10] Therefore, it may cause or increase sinoatrial block, intraatrial block and A-V block or cause intraventricular conduction defects.[4]

The administration of a chelating agent has been used effectively because binding the serum ionizable calcium renders the heart less sensitive to the effects of digitalis.[11-17] Unfortunately, the chelating salt, sodium EDTA (ethylenediaminetetraacetic acid), is hazardous because it may produce severe hypotension, hypocalcemic tetany, severe skin rashes, toxicity of the proximal renal tubular cells and local thrombophlebitis at the site of injection.[11-17] In a previous study it was demonstrated that sodium citrate is a chelating agent in a biologic sense, because it binds serum ionizable calcium.[18] It was demonstrated that digitalis-induced arrhythmias could be corrected quickly by the administration of sodium citrate (Fig. 1). The citrate salt does not cause a drop in systemic pressure as does EDTA. It was also noted that citrate was a specific agent for the diagnosis of digitalis toxicity because it would not convert aconitine-induced or ischemic arrhythmias but always corrected digitalis-induced arrhythmias.[18]

It was noted that the arrhythmias of digitalis toxicity reappeared within a few minutes after cessation of administration of citrate. It was reasoned that if the citrate could be slowly ad-

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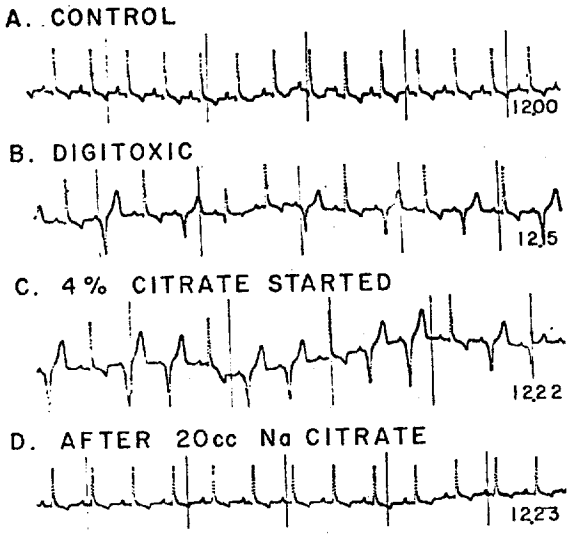

FIG. 1. *Digitalis toxicity treated with sodium citrate.* A, control, showing sinus rhythm. Digoxin was given (0.15 mg./kg.) intravenously. B, 15 minutes later, there is bigeminal rhythm due to frequent ventricular premature systoles. C, 7 minutes later, trigeminal rhythm has occurred. Infusion of 4% sodium citrate was started. D, 1 minute later, after 20 cc. sodium citrate was administered intravenously, sinus rhythm was restored.

ministered over a period of hours until the toxic dose of digitalis was excreted, this would prove to be an effective method for the treatment of arrhythmias of digitalis toxicity.

PROCEDURE

Series A (Control): Twenty dogs were anesthetized with intravenous sodium pentothal. Each animal received a toxic dose of digoxin calculated at 0.15 mg./kg. This dose was administered intravenously, and then sufficient time was allowed for electrocardiographic signs of toxicity to become established. This dose is calculated to be five times that normally given to the adult human per kilogram of body weight. Electrocardiographic monitoring was instituted immediately before injection of the glycoside and was continued for an eight hour period following the onset of arrhythmia. This group of dogs acted as a control series. They did not receive any form of therapy. Those that survived the eight hour period of monitoring were removed to the kennel for a three day observation period (Table 1).

Series B (Treated): Twenty dogs were anesthetized with intravenous sodium pentothal and then given a toxic dose of digitalis as outlined in Series A. A mixture of 50 cc. of a 4% solution of sodium citrate and 50 cc. of a 4% solution of potassium citrate was diluted in 500 cc. of 5% glucose in water and was administered intravenously by slow drip. (Each cc. of solution contained 3.33 mg. of sodium citrate and potassium citrate.) Notation was made of the length of time and the amount of citrate required to convert the digitalis-induced arrhythmia, and the amount of citrate required to maintain regular sinus rhythm over a period of eight hours (Fig. 2 and 3). At the end of this time the animals were sent to the kennel where they received food and water but no other treatment as in the control Series A. Electrocardiograms were recorded on subsequent days.

RESULTS

Series A (Control): Arrhythmias and conduction defects developed in all the animals, and most of them demonstrated multiple disturbances in rhythm and conduction. Thirteen dogs had A-V dissociation and 7 ventricular tachycardia as the major disturbance in rhythm. Other rhythms experienced were sinus arrest in 1 animal and multifocal premature systoles in 2 others. Only one had first degree A-V block as its digitoxic manifestation. Of the total of 20 control dogs which received the standard dose of 0.15 mg. digoxin/kg., 12 died within the first day (Table 1). In this group, therefore, the mortality rate from the administration of a standard dose of digoxin was 60 per cent. The same dose of digoxin was administered to the animals in Series B.

Series B (Treated): As in Series A the animals usually exhibited more than one arrhythmia or conduction defect. The major disturbances of rhythm and conduction which resulted from the digitalis toxicity were A-V dissociation in 15, ventricular tachycardia in 12, supraventricular tachycardia in 1, sinus arrest in 1, and multifocal ventricular premature systoles in 1. The intravenous administration of a variable amount of the sodium-potassium citrate solution (30–90 cc./hr.) converted the arrhythmia or corrected the conduction defect in all 20 animals (Fig. 2 and 3). The average amount of this solution required for conversion was 168 cc.; the range was between 18 and 330 cc. Eighteen of the 20 dogs converted to sinus rhythm; 1 died before sufficient citrate was administered; One converted to a nodal rhythm during the first hour and to sinus rhythm during the first night. The average amount of the sodium-potassium citrate infusion required to correct the arrhythmia and maintain sinus rhythm was 370 cc.; the range was 177 to 650 cc.

Only 2 of the dogs failed to survive following the administration of the sodium-potassium citrate preparation. One of these died 30 minutes following the onset of the intravenous drip before sufficient citrate compound (45 cc.)

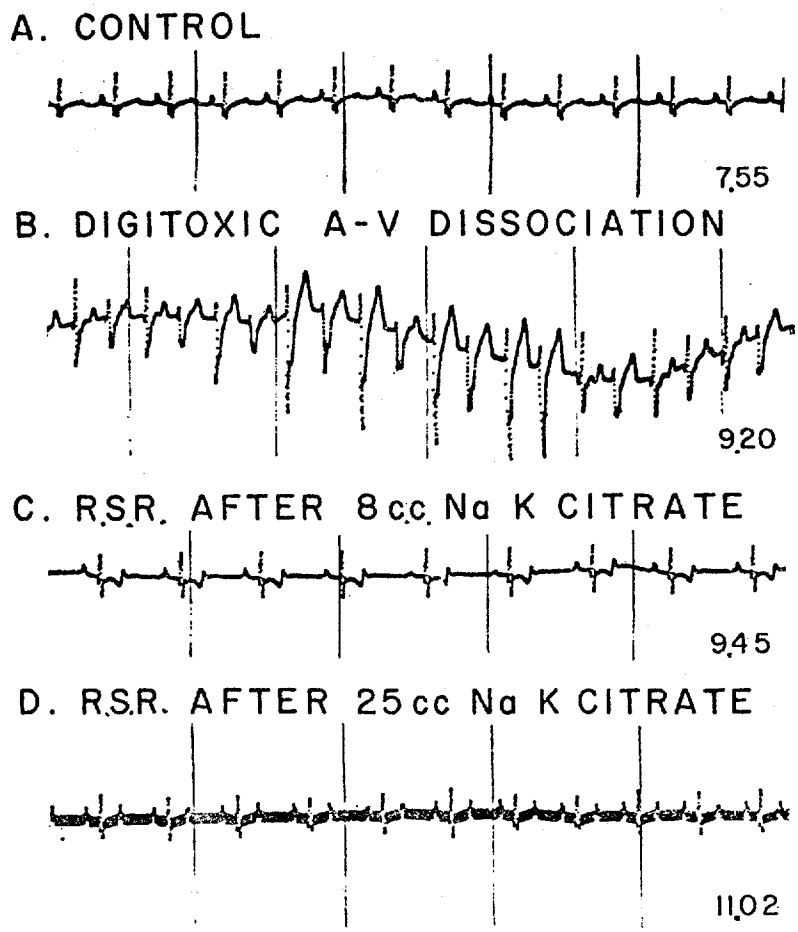

FIG. 2. *Digitalis toxicity treated with sodium-potassium citrate mixture.* A, control, showing sinus rhythm. Digoxin was given (0.15 mg./kg.) intravenously. B, 85 minutes later, there is A-V dissociation with varying aberration. Sodium-potassium citrate solution was started intravenously. C, 25 minutes later sinus rhythm occurred after 8 cc. sodium-potassium citrate. Digitalis effect is evidenced by inverted T waves. D, 77 minutes later, sinus rhythm is restored after a total of 25 cc. sodium-potassium citrate. T waves are now upright.

was administered to correct the arrhythmia. The other died sometime during the night after he was returned to the kennel with normal sinus rhythm (Table I). Because the rhythm was not monitored during the night, the cause of death could not be determined; but it is postulated that it was due to return of the digitalis-induced arrhythmia. The potassium-sodium citrate solution did not cause respiratory or cardiac arrest.

In 5 dogs the citrate level and pH were recorded at the onset of the experiment, at the time of conversion and at the end of infusion. At the time of conversion the citrate level ranged from 3.8 to 5.6 mg.% and at the conclusion of the infusion was 8.8 to 29 mg.%. The serum

TABLE I
Control and Treated Series of Dogs with Digitalis Toxicity

	No. of Dogs	Died	Survived	Mortality Rate (%)	Time of Death (hr.) <2	2–8	8–24
Series A (control)	20	12	8	60	6	2	4
Series B (treated)	20	2	18	10	1	...	1

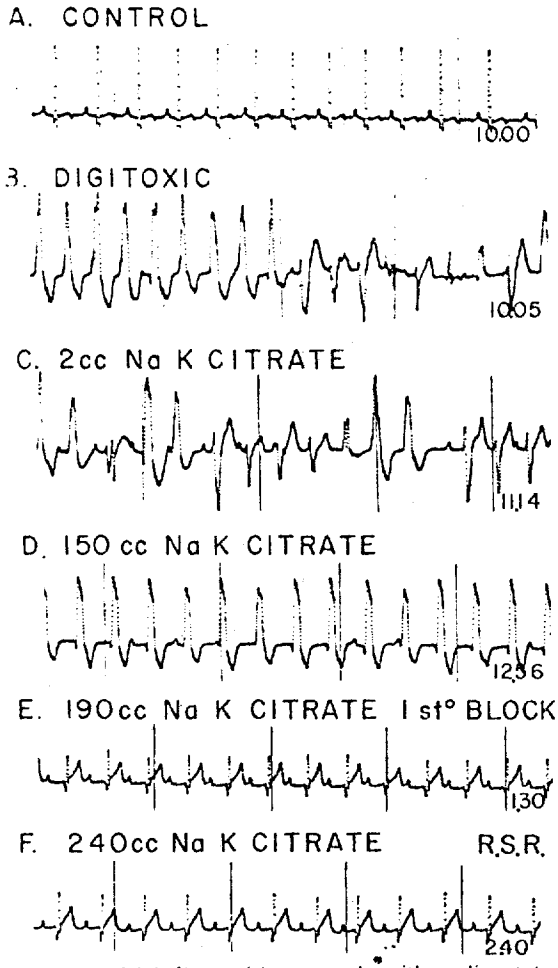

FIG. 3. *Digitalis toxicity treated with sodium-potassium citrate mixture.* A, control, showing sinus rhythm. Digoxin was given (0.15 mg./kg.) intravenously. B, 5 minutes later runs of ventricular tachycardia occurred. C, 69 minutes later A-V dissociation with multifocal ventricular beats is evident. Sodium-potassium citrate was started intravenously. D, 102 minutes later there is ventricular tachycardia. Then 150 cc. sodium-potassium citrate was administered. E, 34 minutes later sinus rhythm with first degree A-V block occurred, and an additional 40 cc. of sodium-potassium citrate was administered. F, 70 minutes later there is sinus rhythm; a total of 240 cc. sodium-potassium citrate was administered.

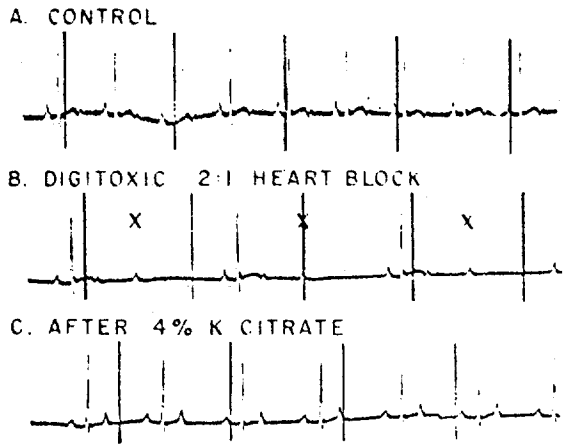

FIG. 4. *Digitalis toxicity treated with 4% potassium citrate.* A, control shows sinus rhythm. Digoxin was given (0.15 mg./kg.) intravenously. B, digitalis toxicity was manifested by 2:1 heart block. Then 4% potassium citrate was started. C, sinus rhythm was restored after intravenous administration of 4% potassium citrate.

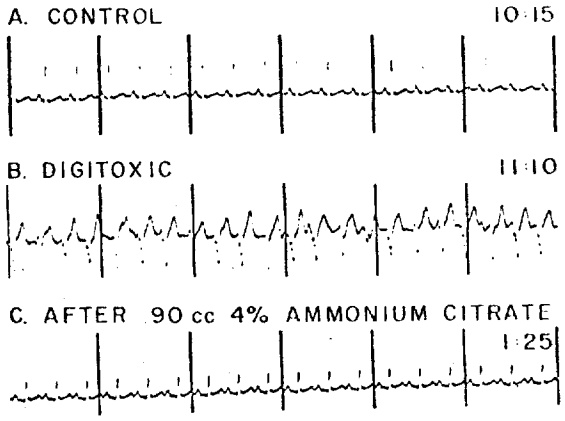

FIG. 5. *Digitalis toxicity treated with 4% ammonium citrate.* A, control shows sinus rhythm. Digoxin was given (0.15 mg./kg.) intravenously. B, 55 minutes later, A-V dissociation with varying aberration occurred. C, 135 minutes later there is sinus rhythm after 90 cc. of 4% ammonium citrate was administered.

potassium and sodium levels remained normal. The serum pH following completion of infusion fell to 7.24 in one animal and rose to 7.64 in one other. One animal that was acidotic (pH = 7.28) at the start became normal (pH = 7.45) during the infusion. The serum pH did not change significantly in the other dogs.

DISCUSSION

These experiments were designed to establish first the mortality rate of a standard toxic dose of digoxin (0.15 mg./kg.) in 20 dogs. Only 8 of the dogs survived this toxic dose which established a 60 per cent mortality rate. The second purpose of this study was to determine the effectiveness of a sodium-potassium citrate solution in the treatment of digitalis-induced arrhythmias. The experiments (Series B) demonstrated that only 2 of the 20 dogs treated with a combination of sodium and potassium citrate died; this lowered the mortality rate to 10 per cent.

Comparison of Various Citrate Salts: In other studies designed to determine the comparative

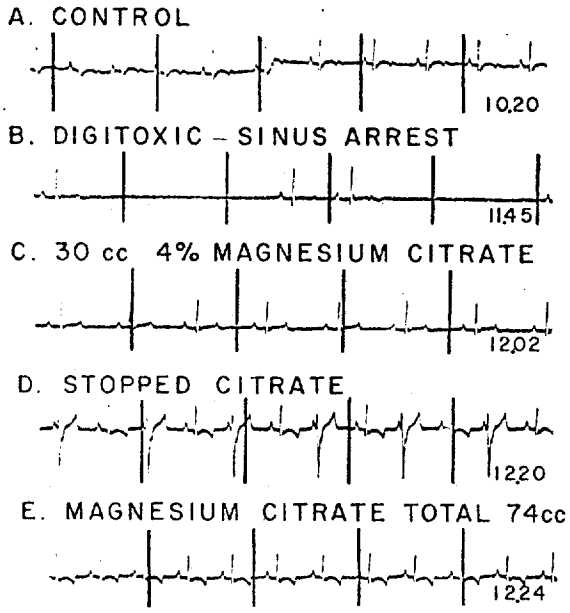

FIG. 6. *Digitalis toxicity treated with 4% magnesium citrate.* A, control shows sinus rhythm. Digoxin was given (0.15 mg./kg.) intravenously. B, 85 minutes later there is sinus arrest; 4% magnesium citrate was started intravenously. C, 17 minutes later sinus rhythm occurred after 30 cc. 4% magnesium citrate. D, 18 minutes after magnesium citrate was stopped. Bigeminal rhythm due to ventricular premature systoles supervened. Magnesium citrate was restarted. E, 4 minutes later sinus rhythm was restored after a total of 74 cc. of 4% magnesium citrate.

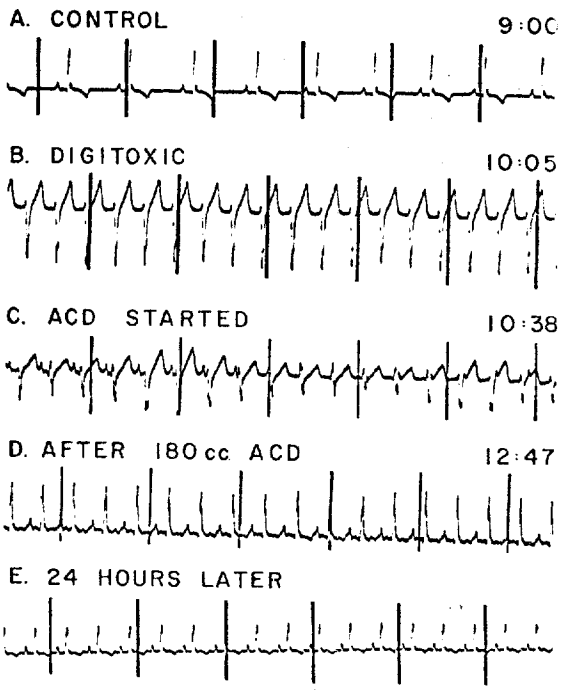

FIG. 7. *Digitalis toxicity treated with acid-citrate-dextrose solution.* A, control shows sinus rhythm. Cedilanid® was given intravenously. B, 65 minutes later there is ventricular tachycardia or supraventricular tachycardia with aberration. C, 33 minutes later A-V dissociation occurred; acid-citrate-dextrose was started. D, 129 minutes later after 120 cc. acid-citrate-dextrose, there is sinus tachycardia with first degree A-V block. E, 24 hours later sinus rhythm was restored.

effectiveness of various citrate compounds for the treatment of digitalis-induced arrhythmias, it was demonstrated that potassium, sodium, ammonium, magnesium and acid-citrate-dextrose were all able to correct digitalis-induced arrhythmias[18] (Fig. 1–7). It was noted that potassium citrate (4% solution) was the most effective compound requiring an average of 50 cc. (Fig. 4). Unfortunately, a 4% solution of ammonium citrate frequently caused tetany, respiratory arrest and peripheral cyanosis which mitigated against its use. Sodium citrate (4% solution) caused initial conversion when an average of 70 cc. was administered (Fig. 1). If large amounts were used, it caused muscle tremors and respiratory arrest. Magnesium citrate (4% solution) was the next most effective citrate salt; it required an average of 100 cc. to cause initial conversion (Fig. 6). Its side effects were hypotension and respiratory arrest in 6 of 8 dogs. Acid-citrate-dextrose solution (ACD solution: sodium citrate 1.32 gm., anhydrous citric acid 0.44 gm., hydrous dextrose 1.47 gm.) was the least effective as it required an average of 124 cc. to cause initial conversion (Fig. 7). The amount used for initial conversion never caused toxic effects.

Therefore, in selecting the most favorable citrate salts for the treatment of digitalis toxicity, sodium and potassium appeared to be most ideal because they caused less toxicity, and smaller amounts were required. Ammonium and magnesium were eliminated from consideration because of their toxic effects. The ACD solution required too great a volume to be a therapeutic agent. Potassium appeared to be the most effective agent for the treatment of digitalis toxicity; but because of its depressing effect on the conduction mechanism which is similar to digitalis, it was reasoned that the amount administered should be limited.[19] Because sodium cannot be administered in large amounts to patients with cardiac decompensation, the volume of this citrate compound also must be limited. It was, therefore, reasoned that a combination of sodium and potassium citrate diluted in 5% glucose in water would be the

ideal method of treating digitalis-induced arrhythmias. This would limit the amount of potassium and sodium in the citrate compound necessary to convert the arrhythmia. The results of using such a solution (Series B, Table 1) have indicated that this is an effective way of treating a digitalis-induced arrhythmia. The mortality rate was reduced from 60 per cent in the control series to 10 per cent in the treated series. This mortality rate might have been improved further, because one of the dogs died before sufficient citrate compound was administered to correct the arrhythmia, and the other animal might have survived had it been monitored during the night and treatment reinstituted when arrhythmias recurred.

Digitalis Toxicity and Serum and Myocardial Electrolytes: The four basic beneficial effects of digitalis[4] are that it will (1) often convert ectopic supraventricular and occasionally ventricular arrhythmias, (2) will block A-V conduction in atrial flutter and fibrillation and thus slow the ventricular rate, (3) will increase the ventricular contractility of the failing heart, and (4) increase the vagus tone. It is a paradox, however, that although digitalis will convert ectopic arrhythmias, it will also induce other more serious arrhythmias. Digitalis may cause any type of ectopic arrhythmia arising in the atria, the A-V node or the ventricles.[4] The most common ectopic arrhythmias due to digitalis are, in order of frequency, premature ventricular systoles, atrial tachycardia and fibrillation, and ventricular tachycardia.[4] Because digitalis retards conduction through the atria, the A-V node or the ventricle, toxic doses often result in conduction defects, such as first, second and third degree heart block and A-V dissociation.[4]

Administration of digitalis requires careful biologic titration because, once digitalization has been achieved, 60 per cent of the toxic dose has already been administered.[6] Also, once toxicity has developed, the patient has received 50 to 80 per cent of the minimum lethal dose.[6] This demonstrates the narrow range of safety in the administration of digitalis. Each patient has an individual requirement, and these requirements are affected by electrolyte imbalances and also the condition of the myocardium. The patient with ischemic heart disease can tolerate less digitalis than the patient with a normal myocardium.[4] There is a delicate balance between the potassium and calcium levels in the myocardium, and an optimal relationship is necessary for normal cardiac action.[4] If the calcium concentration is reduced and potassium raised, there is a decreased irritability of the myocardium. It was formerly believed that diuresis causes digitalis toxicity because mobilization of the digitalis-laden fluids actually causes a redigitalization.[6,7] Recent studies demonstrate that these arrhythmias are due to a potassium loss, and that the heart is more sensitive to digitalis when the serum potassium is reduced.[6,7] At a potassium level of 2 mEq. only 40 per cent of a controlled toxic dose of digitalis results in ventricular tachycardia.[6,7] On the opposite side, it was noted that when potassium was raised to 7 mEq., it required a 240 per cent increase in digitalis to produce toxicity.[6,7] Thus, it was demonstrated that the heart is more sensitive to digitalis when hypokalemia results and suggests that many digitalis-induced arrhythmias may be converted by the administration of potassium.

Potassium has an effect similar to that of digitalis in that it retards conduction through the myocardium and the A-V node. Therefore, it must be used carefully lest it cause more profound conduction disturbances.[10,20] It should not be administered except under careful electrocardiographic control to monitor the effect on conduction. Early warning signs of potassium toxicity are the appearance of electrocardiographic changes such as elevation of the T wave or widening of the QRS.[4] Caution must be maintained once these signs appear.

Rationale of Treating Digitalis Toxicity: Physiologists have shed new light on the cellular action of digitalis and provided us with a more physiologic basis for the treatment of digitalis toxicity. They have demonstrated that there is a reverse shift of sodium and potassium across the cell membrane in various stages of the cardiac cycle, and also that digitalis changes the permeability of the cell membrane so that potassium cannot re-enter the cells as it normally does in the recovery period.[21,22] Szent-Gyorgyi[21] and Hellems et al.[22] have demonstrated that calcium and digitalis both render the cell membrane less permeable to potassium so that it prevents the potassium from re-entering. This lowering of the potassium content within the cell is apt to produce an arrhythmia.[4] The treatment for digitalis toxicity, therefore, should be aimed at improving the ionic exchange of potassium across the cell membrane during the recovery period. Therefore, the rational approach for the treatment of digitalis

toxicity is to remove the digitalis, lower the serum ionizable calcium to improve the membrane permeability, or push potassium into the cell. Unfortunately, it takes time for the digitalis to be excreted, and the administration of intravenous potassium has certain dangers. Therefore, the principle for rapid treatment should be aimed at reducing the serum calcium. This indirectly will allow the potassium to re-enter the cell and, therefore, to balance the electromotive forces on both sides of the cell membrane. Reduction can be accomplished by the judicious use of the chelating agent, sodium EDTA, or citrate salts. This augments the intramyocardial concentration of cellular potassium and balances the electromotive forces at the cell membrane level, which in turn acts to decrease the excitability of the heart muscle cell. The toxic effect of EDTA has now been well demonstrated.[12,23] The principal drawback to its use is vascular collapse. In comparison, vascular collapse does not occur when sodium or potassium citrate is used.

Toxic Effects of Citrate Salts: Citrate compounds in themselves may be toxic. The pathophysiology of citrate toxicity has been documented in its 50 years of use.[24-27] Infused citrate disappears from the plasma as a result of its distribution through the extracellular space, excretion in the urine and metabolic destruction in the liver. Excretion studies have demonstrated that dogs will excrete 40 per cent of the infused citrate in the urine, while man eliminates only 20 per cent by this route.[24] Man metabolizes citrate mainly in the liver. If liver function is adequate, the administration of 1 mg. of citrate per kilogram of body weight per minute will cause a rise of serum citrate of 12.5 mg./100 cc. A toxic level of citrate is above 50 mg.%. In our series of dogs the citrate was not found to rise to toxic levels in any instance. It rose to 29 mg.% on only one occasion. Actually, the average amount of sodium-potassium citrate used in the course of these experiments was only 2 gm. of citrate as a salt. Therefore, it is evident that the amount of citrate required to treat the average animal was comparable to the amount of citrate used in man given 5 units of blood over an 8 to 10 hour period. Man can withstand such an amount of citrate in a blood transfusion without its causing toxicity.

Signs of citrate toxicity consist of muscle twitching or convulsions in the unanesthetized patient, electrocardiographic changes—particularly lengthening of the Q-T interval which may progress to cardiac asystole, systemic arterial hypotension resistant to blood transfusion or vasopressor agents—and possibly prolongation of the clotting time.[24] Bunker et al.[28] demonstrated direct evidence of cardiac depression in the dog by strain-gauge measurements consisting of a rise in central venous pressure and end-diastolic pressure in the left ventricle. However, their reported rate of administration of citrate was at least 10 times greater than that which we used in treating digitalis toxicity (4 mg./kg./min., as compared to 0.4 mg./kg./min.). Circulatory depression may occur at blood levels of citrate greater than 47 mg.%. The highest blood level we encountered was 29 mg.%.

Summary

Sixty per cent of a control series of the dogs died from a standard toxic dose of digitalis. In a series rendered toxic by a similar dose of digitalis, only 10 per cent died when they were treated with the combination of sodium-potassium citrate salts.

A combination of sodium and potassium citrate salts administered intravenously is effective for the treatment of digitalis-induced arrhythmias.

Acknowledgment

We wish to thank Harry Mier, E. D. Mitchell, and the Jay Paley, Maurice Katleman, Anna Robbins and George Cordingly Memorial Funds for financial support to this study. We are indebted to Willie Davis, Willis Curtis Pea, Myles Prevost, and Jeanne Bloom for their technical assistance.

We would like to thank Burroughs Wellcome & Co. for supplying us with digoxin (Lanoxin).

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Influence of various potassium salts on the toxicity of digitalin on guinea pig heart

By R. Hazard, J. Hazard and J. Thouvenot

(Received June 7, 1955)

The effect of electrolytes on cardiac activity has long been known. Experiments on the isolated and perfused heart show that any change in the composition of the Ringer solution causes grave cardiac perturbations. The action of the cardiants, especiallythe digitalics, can be modified by an electrolytic perturbation. It is known, in fact, that the action of the digitalics on the myocardium is increased by calcium (J. Labarre and J. van Heerswynghels (7)) and that it is influenced also by the potassium concentration, by its rise as well as its lowering.

The lowering of the potassium concentration strengthens the action of the digitalics:

The absence of potassium in the perfusion liquid of a duck embryo heart increases the effects of Lanatoside C (M. Friedman and R. Bine, 2).

The treatment of cardiac edema with cation exchange resins permitted Cl. Laroche, J. Cruveillier, J. Hazard and J. Truffert (8) to find, when the kaliemia was reduced, a remarkable action of doses of digitalin which had previously been ineffective in the same patients. The digitalics and the catiopexic resins prescribed separately had also been ineffective. This remarkable action of the digitalin can in part be explained by the removal of the obstacle that the edemata constituted, but also by the lowering of the kaliemia brought about in those patients. Similar findings were obtained under the same conditions by some American authors, who reported an increase in the activity and in the toxicity of digitalin (S. H. Hay and E. J. Wood (3); S. Sacks (11)).

Under different conditions, but also in cases of hypokaliemia, B. Lown, H. Salz-

berg, C. D. Enselberg and R. E. Weston (9) observe increased sensitivity to digitalin after administration of mercurial diuretics, desocy-corticosterone acetate or insulin and glucose.

When lowering the degree of kaliemia in the dog by means of an artificial kidney, B. Lown, J. M. Weller, N. Wyatt, R. Hoigne and J. P. Merril (10) found a 60% increase of the toxicity of acetyl strophanthidin.

Increase of the potassium concentration opposes the action of digitalics:

It is known that potassium can combat certain effects of digitalin, J. J. Sampson and E. M. Anderson (12), C. D. Enselberg, H. G. Simmons and A. A. Mintz (1) proposed treating certain arrhythmias caused by digitalin with this ion. These clinical notions were confirmed on an experimental level. H. Friedman and R. Bine (2) found that an excess of potassium inhibits the effects of lanatoside C on the heart of duck embryo.

A first series of tests made on reducing the toxicity of digitalin in the guinea pig by using various potassium salts permitted two of us to conclude (5, 5) that these salts, under specific experimental conditions. afford a limited but real protection against the toxicardiac effect of glucoside. As these studies were later extended to other potassium salts, we shall compile here the results of several experiment series, subjecting them to the statistical treatment at present in use in pharmacodynamics.

Technique used

Homogeneous lots of guinea pigs are given, after anesthesia with urethan (at the dose of 1 g per kg intraperitoneally), an intra-jugular injection of digitalin solution (diluted to 1 p. 10,000 in physiologic solution) either pure or added with potassium salt at the tested concentrations. With a Jouvelet electric pump a regular perfusion of the solutions is made, constant in all experiments (1.13 cc per kg of animal and per minute).

In each case the perfusion is continued to heart failure. The volume injected gives the quantity of digitalin used, and from it the median lethal dose per kg is deduced. The injection time constitutes an additional element of appraisal of the toxicity.

As the first findings with potassium chloride had shown that this salt reduces the toxicity of digitalin to a certain optimum degree, we investigated the reasons for this protection and considered in particular the possible intervention of the antagonism potassium/calcium. The latter element, as is known (7), increases the toxicity of glucoside. Its inactivation by the anion joined to the potassium should, one would think, further increase the protective effect of the cation.

We therefore tried potassium phosphate, citrate, fumarate, maleate and oxalate, and complexon II as K salt, at various concentrations which correspond to the same quantity of potassium arbitrarily chosen: 0.110 g and 0.157.

The mode of inactivation of the calcium is not doubt quite different depending on the salt used - relative insolubilization in a protidic medium, de-ionization, etc.

Results obtained

These results were treated statistically.

1. We verified that in each lot of guinea pigs the distribution of the individual values of the toxicity and duration of perfusion followed the normal law. For each series of n guinea pigs we calculated the standard deviation $sigma_M$ from the mean m given by the following formula:

$$sigma_M = \text{square root of } S\ IGMA\ (x-m)^2 \text{ over } n(n-1)$$

The term $SIGMA\ (x-m)^2 = SIGMA\ x^2 - T^2/n$ where $T = SIGMA\ x$

From $sigma_M$ one can calculate the confidence limits which involve $sigma_M$ and the value of the t found in Student's table (1*) for a number n of animals and at the chosen threshold of probability, namely here $P = 0.05$. These limits are given by the expression $m \pm sigmat_M$.

These limits thus calculated have the following meaning: Under these conditions the chances are 95% favorable for affirming that the true value of the toxicity or the duration of the perfusion, in itself unknown, is comprised between these limits (Table I).

Table I. Influence of different potassium salts on the toxicity of digitalin.

No. of animals n	Perfused solutions	A. Toxicity of digitalin in mg/kg Mean	$sigma_M$	Conf. limits at P=0.05	B. Perfusion time (min) Mean	$sigma_M$	Conf. limits at P = 0.05
20	Digitalin alone (2 series cumulated)	1.60	± 0.0265	± 0.055	14.17	± 0.008	± 0.21
10	Dig.+ClK 0.10 g%	1.67	± 0.049	± 0.11	14.85	± 0.40	± 0.90
10	Dig.+ClK 0.21 g%	2.02	± 0.065	± 0.15	18.00	± 0.59	± 1.32
10	Dig.+ClK 0.25 g%	2.02	± 0.063	± 0.14	18.10	± 0.52	± 1.16
10	Dig.+ClK 0.30 g%	2.02	± 0.063	± 0.14	19.00	± 0.45	± 1.02
10	Dig.+ClK 0.35 g%	2.02	± 0.087	± 0.20	17.85	± 0.75	± 1.71
10	Dig.+ClK 0.40 g%	1.72	± 0.093	± 0.21	15.15	± 0.85	± 1.92
5	Dig.+ClK 0.60 g%	1.47	± 0.24	± 0.67	12.60	± 0.66	± 1.82
10	Dig+monopotassium phosphate at 0.615 g% (K^+ = 0.157 g%)	1.78	± 0.071	± 0.61	16.10	± 0.66	± 1.48
10	Dig.+dipotassium phosphate at 0.382 g% (K^+=0.157 g%)	2.10	± 0.062	± 0.14	18.60	± 0.55	± 1.24
[illegible]	Dig. K citrate at 0.142 g% (K^+ = 0.551 g%)	2.35	± 0.59	± 0.13	21.25	± 0.71	± 1.59
10	Dig.+K citrate at 0.30 g% (K^+ = 0.110 g%)	2.58	± 0.17	± 0.18	23.03	± 0.51	± 1.16
5	Dig.+ K citrate at 0.425 g% (K^+ = 0.156 g%)	1.90	± 0.043	± 0.12	15.86	± 1.61	± 4.45
10	Dig. + K fumarate (K^+ = 0.110 g%)	1.79	0.104	± 0.24	16.05	0.90	± 2.03
10	Dig. + K fumarate (K^+ = 0.157 g%)	1.93	0.04	± 0.091	17.23	0.36	± 0.81
10	Dig. + K maleate (K^+ = 0.110 g%)	2.18	0.062	± 0.14	18.85	0.52	± 1.16
10	Dig. + K maleate (K^+ = 0.157 g%)	1.72	0.048	± 0.11	15.2	0.42	± 0.94
10	Dig.+ K oxalate 0.37 g% (K^+ = 0.157 g%)	2.08	0.122	± 0.28	18.24	0.81	± 1.84
10	Dig.+Complexon II 0.21 g% (K^+ = 0.110 g%)	2.24	0.055	± 0.12	20.05	0.48	± 1.08

By comparison we have studied the following two series with sodium citrate

10 Dig.+Na citrate 0.131 g%	1.77	0.041	± 0.93	15.80	0.38	± 0.86
10 Dig.+Na citrate 0.30 g%	1.84	0.074	± 0.17	16.25	0.60	± 1.36
10 Dig.+Na citrate 0.393 g%	1.81	0.069	± 0.16	16.2	0.67	± 1.51

2. To test the degree of significance of the difference between the two means of the lots to be compared in pairs (case of independent samples) we determined the value of the Student-Fisher t.

$$t = \frac{m_1 - m_2}{\sqrt{\frac{\Sigma (x_1 - m_1)^2 + \Sigma (x_2 - m_2)^2}{n_1 + n_2 - 2} \left(\frac{1}{n_1} + \frac{1}{n_2}\right)}}$$

where m_1 and m_2 are the means to be compared, n_1 and n_2 the number of animals in each lot, and $SIGMA_1 (x_1-m_1)^2$ represents the sum of the squares of lot 1, $SIGMA_2 (x_2-m_2)^2$ that of lot 2.

Knowing the value of t calculated, one can compare it with the value t found in the table for the corresponding number $n_1 + n_2 - 2$ degrees of freedom. It follows that one can situate the level of probability P corresponding to the comparison which is indicated in Table II with its significance.

Interpretation of the results

a) The addition of potassium chloride (Fig. 1), if its concentration is sufficient, reduces the toxicity of digitalin.

If the concentration is too low (0.10 g%), this reduction is not great: The median lethal dose per kg passes from 1.60 ± 0.055 for digitalin alone to 1.67 ± 0.11; the difference is not significant (P = 0.2).

The addition of ClK at 0.21 g% causes a very significant difference from digitalin alone. The LD_{50} passes from 1.60 ± 0.055 to 2.02 ± 0.15 (P = 0.001) and in relation to digitalin plus ClK 0.10 g% again P = 0.001.

This seems to be the minimum effective dose of ClK, very close also to the optimum dose, as higher concentrations (0.25 g = 0.30 g - 0.35 g) do not improve the effect obtained (see in fig. 1 the curve adjusted to the distribution (++++)); the optimum action corresponds to 0.25 to 0.28 g% of ClK. At higher doses of ClK (0.40 and 0.60), the minimum lethal dose of the mixture becomes very similar again to that of digitalin or even falls below it, probably due to the toxicity of

potassium itself.

b) The activity of monopotassium phosphate is clear: The LD_{50} passes from 1.60 ± 0.055 for digitalin alone to 1.78 ± 0.61 for the mixture digitalin + monopotassium phosphate 0.61 g% (K^+ = 0.157 g%) for a probability theshold $P < 0.001$.

Fig. 1. Toxicity of digitalin (in mg/kg) alone and in the presence of potassium chloride at increasing concentrations. The perfusion time is shown in broken lines. Above each rectangle constituting the histogram we have indicated the confidence limits of the mean of each sample given in Table I. The number of animals in each series is stated in Table I. To reduce the length of the figure in the vertical direction we have cut it horizontally.

We tried to trace a curve adjusted to the distribution (+++++). It can be seen that the optimum antitoxic action of ClK toward digitalin corresponds to ClK concentrations in the neighborhood of 0.25 to 0.28 g%.

Duration of perfusion in min

Toxicity of digitalin mg/kg

The action of dipostassium phosphate is less clear: The LD_{50} passes from 1.60 ± 0.055 for digitalin alone to 2.10 ± 0.14 after addition of dipotassium phosphate to the digitalin, at a probability threshold $P = 0.02$.

Can the favorable action of the K ion, within certain limits, be attributed to its antagonism to the calcium ion? If so, the inactivation of the latter should favor still more the detoxicating action of the potassium toward the digitalin.

Fig. 2. Compared action of the citrates of K and of Na on the toxicity of digitalin indicated as in Figure 1 by the toxicity in mg/kg and the volume of the toxic perfusion used. The general indications are the same as given for Figure 1. It is clearly evident that if the antitoxic action proper to the citrate ion appears for the Na citrate, the potassium ion considerably reinforces this effect.

Perfusion time in min - Toxicity of digitalin mg/kg

Action of K citrate - Action of Na citrate

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c) Potassium oxalate (2*) (K^+ = 42.4%) in solution at 0.37 g%, which causes it to precipitate, does not protect more (LD_{50} = 2.08 ± 0.28) than potassium chloride at the optimum dose of 0.21 g% (LD_{50} = 2.02 ± 0.15) no doubt by reason of the toxicity of the oxalate ion itself for the heart (R. Hazard, L. Wurmser and J. Cheymol, 4).

d) Potassium citrate (Fig. 2) reduces the toxicity of digitalin more than the chloride for salts containing the same quantity of element K^+ (0.110 g%). There is a clear difference between the LD_{50} of digitalin alone (1.60 ± 0.055) and that of the mixture digitalin + potassium citrate at 0.30 g% (2.58 ± 0.38) at the probability threshold P = 0.001; this is true also for the perfusion time.

This concentration of potassium citrate at 0.30 g% seems to be close to the optimum concentration, for if one brings the dose of K citrate to 0.425 g%, the LD_{50} drops to 1.90 ± 0.12, a significant deviation from the LD_{50} obtained with K citrate at 0.30 at the probability threshold P = 0.05.

After addition of sodium citrate (Fig. 2) at 0.30 g%, the LD_{50} of digitalin passes from 1.60 ± 0.055 to 1.84 ± 0.17; it seems that the citrate ion exerts an action of its own which increases the lethal dose od digitalin; in fact: (1) At equal weight of K^+ (0.110 g%) the potassium citrate increases the lethal dose in relation to ClK; (2) sodium citrate by itself, independently of the potassium, increases the lethal dose.

Lastly between sodium citrate (LD_{50} = 1.84 ± 0.7) and potassium citrate (LD_{50} = 2.58 ± 0.38) there is a significant difference, due evidently to the presence of K^+, at the probability threshold P = 0.01. In the potassium citrate molecule the potassium exerts a specific action.

e) The addition of potassium fumarate (3*) (Fig. 3) at different concentrations (K^+ = 0.110 and 0.157 g %) does not validly modify the toxicity of digitalin alone.

f) Potassium maleate (Fig. 3) at K^+ = 0.110 g % reduces the toxicity of digitalin very clearly: The LD_{50} of digitalin passes from 1.60 ± 0.055 to 2.18 ± 0.14 at the probability threshold P = 0.001. This K maleate concentration seems to be close to the optimum dose, for if it is raised, the LD_{50} decreases to 1.72 ± 0.11

(K^+ = 0.157 g%).

g) The use of a K salt of complexon II (4*) at 0.21% (K^+ = 0.110%) permits raising the LD_{50} of digitalin from 1.60 ± 0.055 to 2.24 mg/kg ± 0.12, with a very strong difference (P = 0.001).

Fig. 3. Action of K fumarate and of K maleate at different concentrations on the toxicity of digitalin. The control lot, digitalin alone, is placed to the left of each histogram. As in figures 1 and 2, we have cut the figure off in the vertical direction to reduce its dimensions without reducing the scale. The numbers over each rectangle indicate the confidence limits of the mean (see Table I).

It can be seen that K fumarate and maleate behave differently. The maleate has an optimum action for a narrow range of concentrations; for the K fumarate lot we have perhaps not yet reached the optimum effect.

Table II. Comparisons between the lots; degree of significance of the difference between the means of each lot.

Degrees of freedom $n_1 + n_2 - 2$	A. Toxicity of digitalin (10^{-4})	t calc.	P	Sign. diff.	B. Perfusion time (in min) t calc.	P	Significance of difference
	1. Digitalin and potassium chloride						
18	Digitalin alone Dig. + ClK 0.10%	1.27	0.2	none	1.66	0.2>P>0.1	none
18	Dig. + ClK 0.10% Dig. + ClK 0.21%	4.37	0.001	very great	4.38	0.001	very great
18	Dig. + CLK 0.21% Dig. + ClK 0.25%			none	0.129	0.9	none
18	Dig. + Clk 0.25 % Dig. + ClK 0.30%	0.18	0.9	none	1.31	0.2	none
18	Dig. + ClK 0.30% Dig. + ClK 0.35%	1.08	0.3	none	1.30	0.2	none
18	Dig. + ClK 0.35% Dig. + ClK 0.40%	2.34	0.05	substantial	2.38	0.05	substantial
18	Dig. + ClK 0.40% Dig. + ClK 0.60%	2.184	0.05	substantial	2.83	0.02	quite strong

2. Digitalin and monopotassium phosphate (K = 157 mg%)

18	Digitalin alone Dig. + PO_4KH_2	6.08	<0 001	very great	2.81	0.02	great

3. Digitalin and dipotassium phosphate (K = 157 mg%)

18	Digit. alone and Digit. + PO_4K_2H	2.60	0.02	quite great	7.48	0.001	great

4. Digitalin and potassium citrate

18	Digitalin alone Dig. + K citrate 0.142 g%	14.58	<0.001	very great	9.51	0.001	very great
18	Dig. + K citrate 0.142 g% Dig. + K citrate 0.30 g%	1.27	>0.2	small	2.05	>0.05	very great
18	Dig. + K citrate 0.30 g% Dig. + K citrate 0.42g%	2.74	<0.02	great	6.73	0.001	very great

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5. Digitalin and sodium citrate (to be compared with 4)

	Digit. alone and Dig.+Na citrate 0.131 g%	3.69	0.01-0.001	very great	3.65	0.01-0.001	very great
18	Digit.+ Na citrate 0.131 g% Digit.+Na citrate 0.30 g%	1.09	<0.3	small	0.49	>0.6	none
18	Digit.+ Na citrate 0.30 g% Digit.+ Na citrate 0.39 g%	0.31	-	none	0.054	-	none

5bis. Comparisons between K and Na citrates

18	Dig.+ K citrate 0.30 g% Dig.+ Na citrate 0.30 g%	3.97	0.01	great	8.13	0.001	very great
13	Dig.+ Na citrate 0.39 g% Dig.+ K citrate 0.42 g%	0.91	0.4	none	0.82	0.4	none

6. Digitalin and potassium fumarate

	Digitalin alone						
18	Dig.+ K fumarate	1.90	0.1	small	2.13	0.05	substantial
	Dig.+ K fumarate (K^+ = 0.110 g%)						
18		1.27	0.2	none	1.22	0.2	none
	Dig.+ K fumarate (K^+ = 0.157 g%)						

7. Digitalin and potassium maleate

18	Digitaline alone						
	Dig.+ K maleate (K^+ = 0.110 g%)	7.65	0.001	very strong	8.28	0.001	very strong
	Dig.+ K maleate (K^+ = 0.110 g%)						
18		5.00	0.001	very strong	5.53	0.001	very strong
	Dig.+ K maleate (K^+ = 0.157 g%)						

8. Digitalin and potassium oxalate

	Digitalin alone						
18	Dig.+ K oxalate (K^+ = 0.157 g%)	3.96	$0.01 > P >$	strong	5.06	0.001	very strong

9. Digitalin and Complexon II acid

	Digitalin alone						
18	Dig.+ Complexon	10.27	0.001	very strong	11.1	0.001	very strong

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Conclusions

The various potassium salts used all protect the guinea pig heart against the toxic effects of digitalin.

Their activity varies according to the anion of the salt; it passes through a maximum and then decreases beyond an optimum concentration.

The most effective is potassium citrate, which causes the median lethal dose of glucoside to pass from 1.60 to 2.58 mg/kg.

In decreasing order of protection and according to the best results recorded, we have: the potassium salt of complexon II (2.24), the maleate (2.18), the phosphate (2.10), the oxalate (2.08), the chloride (2.02), the fumarate (1.93). The inactivation of the calcium plays a part, but it is undoubtedly obtained by a number of not very well defined processes in a complex medium and it does not seem possible to specify those which establish the best condi-

tions.

Despite the strict mathematical treatment of the values presented, it is evident that they are not absolute and should be supplemented by a much broader experimentation. But even if they represent only an approximation, they permit us nevertheless to attribute to the potassium ion a relative detoxicating action on digitalin.

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(Footnotes)

(1*) See Statistical Table of R. A. Fisher and F. Yates, Oliver and Boyd, London 1953.

(2*) The mean toxicity of this oxalate itself in solution at 0.37 g% is 33.28 ± 2.15 cc/kg. Perfusion time 29.66 ± 1.85 minutes (mean ± sigma/root n calculated on a series of 10 guinea pigs).

(3*) The mean toxicity of K fumarate for a solution at 0.5 g% K is 0.214 ± 0.044 cc/kg. Perfusion time 37.9 ± 0.95 minutes (mean ± sigma/root n calculated on 9 guinea pigs).

Likewise for the maleate at 0.5 g% K the mean toxicity is 0.182 ± 0.016 cc/kg.

Perfusion time 32.70 ± 0.25 minutes (mean ± sigma/root n calculated on 10 guinea pigs).

(4*) The mean toxicities of complexon II acid for solutions corresponding to K^+ 0.21 g% and K^+ 0.30 g% were, respectively, for the first 49.26 ± 0.20 cc/kg (perfusion time 22.90 ± 2.30 minutes) (mean ± sigma/root n calculated on 10 guinea pigs); for the second, 25.15 ± 2.81 cc/kg (perfusion time 32.70 ± 0.25 minutes (mean ± sigma/root n calculated on 12 guinea pigs).

Translated by Carl Demrick Associates, Inc./LH/db

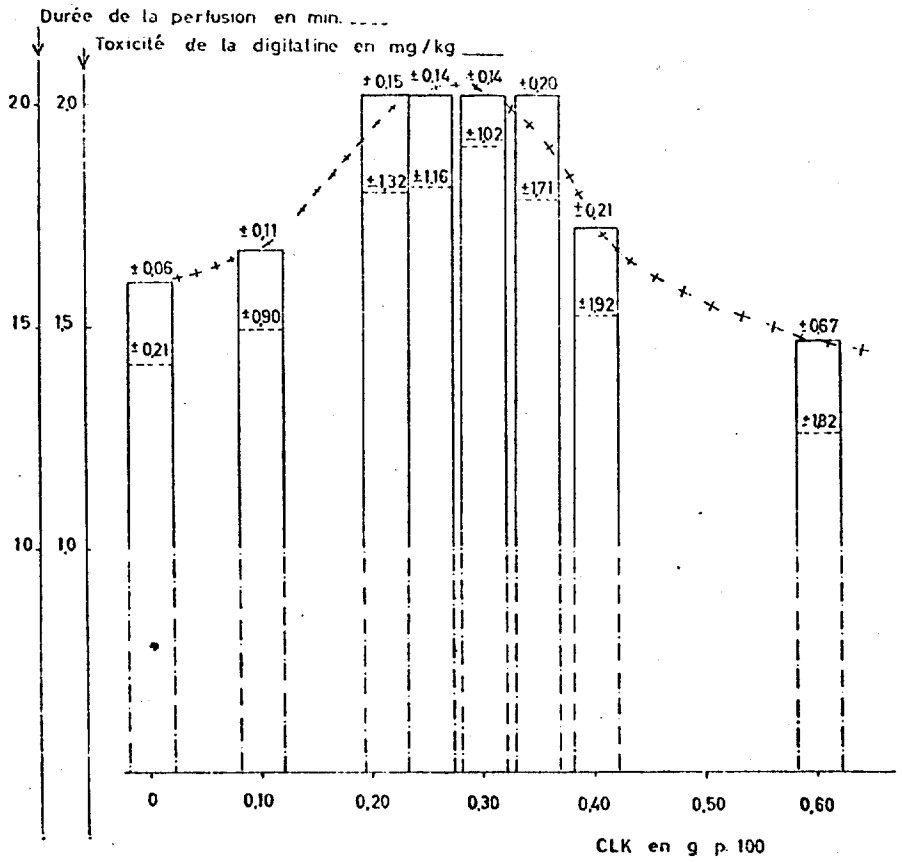
Durée de la perfusion en min. ----
Toxicité de la digitaline en mg/kg ____
20
15
10
2,0
1,5
1,0
±0,06
±0,21
±0,11
±0,90
±0,15
±1,32
±0,14
±1,16
±0,14
±1,02
±0,20
±1,71
±0,21
±1,92
±0,67
±1,82
0
0,10
0,20
0,30
0,40
0,50
0,60
CLK en g p. 100

FIG. 1

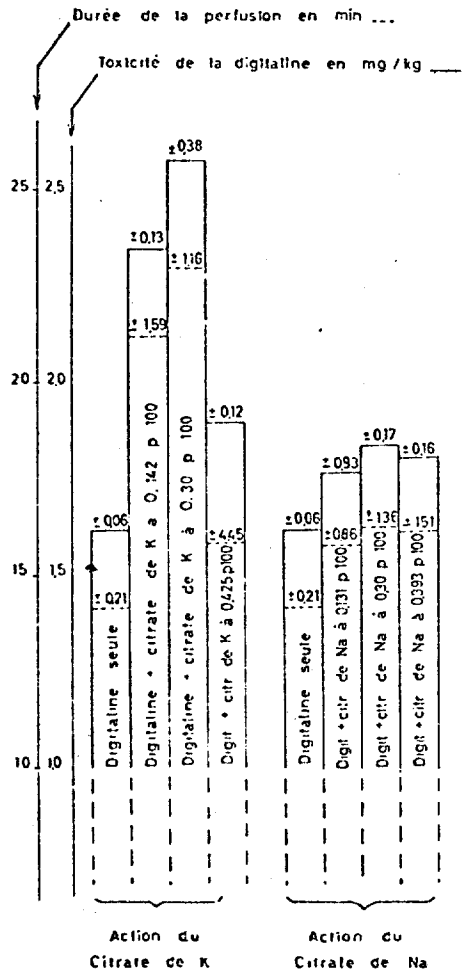
Durée de la perfusion en min ...
Toxicité de la digitaline en mg/kg ___
25
20
15
10
2,5
2,0
1,5
1,0
±0,06
±0,21
±0,13
±1,59
±0,38
±1,16
±0,12
±4,45
Digitaline seule
Digitaline + citrate de K à 0,142 p 100
Digitaline + citrate de K à 0,30 p 100
Digit + citr de K à 0,425 p 100
±0,06
±0,21
±0,93
±0,86
±0,17
±1,36
±0,16
±1,51
Digitaline seule
Digit + citr de Na à 0,131 p 100
Digit + citr de Na à 0,30 p 100
Digit + citr de Na à 0,393 p 100
Action du Citrate de K
Action du Citrate de Na

FIG. 2

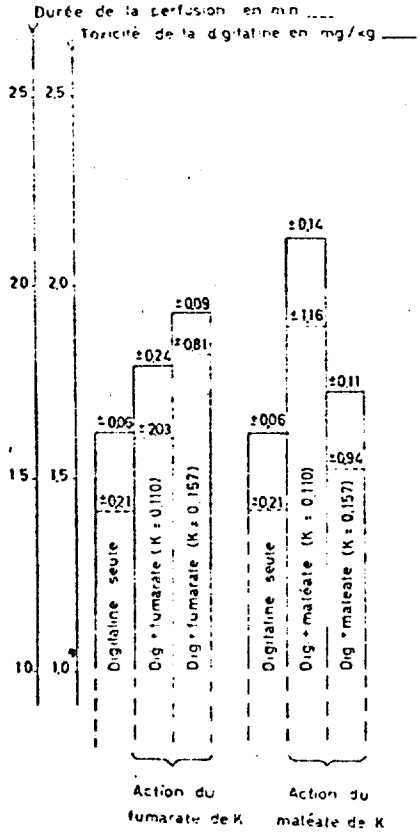
Durée de la perfusion en min ...
Toxicité de la digitaline en mg/kg ___
25
20
15
10
2,5
2,0
1,5
1,0
±0,06
±0,21
±0,24
±2,03
±0,09
±0,81
Digitaline seule
Dig + fumarate (K = 0,110)
Dig + fumarate (K = 0,157)
±0,06
±0,21
±0,14
±1,16
±0,11
±0,94
Digitaline seule
Dig + maléate (K = 0,110)
Dig + maleate (K = 0,157)
Action du fumarate de K
Action du maléate de K

FIG. 3

Arch. int. pharmacodyn., 1956, CV, No 1-2. 530

TRAVAIL DU LABORATOIRE DE PHARMACOLOGIE DE LA FACULTÉ DE MÉDECINE
ET DE L'INSTITUT NATIONAL D'HYGIÈNE

Potassium Citrate

INFLUENCE DE DIVERS SELS DE POTASSIUM SUR LA TOXICITÉ DE LA DIGITALINE SUR LE CŒUR DE COBAYE

PAR

R. HAZARD, J. HAZARD ET J. THOUVENOT

(Travail reçu le 7-6-1955).

L'influence des électrolytes sur l'activité du cœur est connue depuis longtemps. Les expériences sur le cœur isolé et perfusé démontrent que toute modification dans la composition du liquide de Ringer provoque de graves perturbations cardiaques. L'action des toni-cardiaques, des digitaliques en particulier, peut être modifiée par une perturbation électrolytique. On sait en effet que l'action des digitaliques sur le myocarde est accrue par le calcium [J. LABARRE et J. VAN HEERSWYNGHELS, (7)] et qu'elle est influencée aussi par le taux du potassium, aussi bien par son élévation que par son abaissement.

L'abaissement du taux du potassium renforce l'action des digitaliques :

L'absence de potassium dans le liquide de perfusion d'un cœur d'embryon de canard augmente les effets du Lanatoside C [M. FRIEDMAN et R. BINE, (2)].

Le traitement d'oedèmes cardiaques par des résines échangeuses de cations a permis à Cl. LAROCHE, J. CRUVEILLIER, J. HAZARD et J. TRUFFERT (8) de constater, lorsque la kaliémie est abaissée, l'action remarquable de doses de digitaline qui précédemment étaient inefficaces chez les mêmes malades. Les digitaliques et les résines catiopexiques prescrits isolément étaient également inefficaces. Cette action remarquable de la digitaline peut être expliquée en partie par la levée de l'obstacle que constituent les oedèmes, mais aussi par l'abaissement de la kaliémie provoqué chez ces malades. Des constatations analogues ont déjà été faites dans les mêmes conditions par certains auteurs américains

qui ont signalé un accroissement de l'activité et de la toxicité de la digitaline [S. H. HAY et E. J. WOOD, (3); S. SACKS, (11)].

Dans des conditions différentes, mais aussi dans des cas d'hypokaliémie, B. LOWN, H. SALZBERG, C. D. ENSELBERG et R. E. WESTON (9) observent une sensibilité accrue à la digitaline après administration de diurétiques mercuriels, d'acétate de désoxy-corticostérone ou d'insuline et de glucose.

En abaissant le taux de la kaliémie chez le chien à l'aide d'un rein artificiel, B. LOWN, J. M. WELLER, N. WYATT, R. HOIGNE et J. P. MERRIL (10) ont constaté une augmentation de 60 p. 100 de la toxicité de l'acétylstrophantidine.

L'augmentation du taux du potassium s'oppose à l'action des digitaliques :

On sait que le potassium peut combattre certains effets de la digitaline : J. J. SAMPSON et E. M. ANDERSON (12), C. D. ENSELBERG, H. G. SIMMONS et A. A. MINTZ (1) ont proposé de traiter par cet ion certains troubles du rythme provoqués par la digitaline. Ces notions cliniques ont été confirmées sur le plan expérimental : H. FRIEDMAN et R. BINE (2) ont constaté qu'un excès de potassium inhibe les effets du lanatoside C sur le cœur de l'embryon de canard.

Une première série de recherches faites sur la diminution par divers sels de potassium de la toxicité de la digitaline chez le Cobaye avait permis à deux d'entre nous de conclure (5), (6) que ces sels, dans des conditions expérimentales définies, apportent une protection limitée mais réelle contre l'effet toxicardiaque du glucoside. Ces recherches ayant été ensuite étendues à d'autres sels de potassium, nous rassemblons ici les résultats de plusieurs séries d'expériences en les soumettant à l'étude statistique actuellement en usage en pharmacodynamie.

TECHNIQUE UTILISÉE

Des lots homogènes de cobayes reçoivent, après anesthésie par l'uréthane (à la dose de 1 g par kg par voie intra-péritonéale), une injection intra-jugulaire de solution de digitaline (diluée à 1 p. 10.000 dans le soluté physiologique) soit pure, soit additionnée du sel de potassium aux concentrations étudiées. Une pompe électrique de Jouvelet permet de faire une perfusion régulière des solutions, et constante dans toutes les expériences (1,13 cm³ par kg d'animal et par minute).

Dans chaque cas la perfusion est poursuivie jusqu'à l'arrêt cardiaque. Le volume injecté donne la quantité de digitaline utilisée et l'on en

déduit la dose mortelle moyenne (D. M. M.) par kg. La durée de l'injection constitue un élément supplémentaire d'appréciation de la toxicité.

Les premières constatations faites avec le chlorure de potassium ayant montré que ce sel diminue jusqu'à un certain degré optimal la toxicité de la digitaline, nous avons cherché les raisons de cette protection et envisagé notamment l'intervention possible de l'antagonisme potassium/calcium. Ce dernier élément accroît en effet, on le sait (7) la toxicité du glucoside. Son inactivation par l'anion uni au potassium devait, on pouvait le penser, augmenter encore l'effet protecteur du cation.

Nous avons donc essayé le phosphate, le citrate, le fumarate et le maléate, l'oxalate de potassium, le complexon II sous forme de sel de K à diverses concentrations qui correspondent à la même quantité de potassium choisie arbitrairement : 0,110 g et 0,157 g p. 100.

Le mode d'inactivation du calcium est sans doute fort différent suivant le sel utilisé : insolubilisation relative dans un milieu protidique, désionisation, etc.

Résultats obtenus

Les résultats précédents ont été traités statistiquement :

1. Nous avons vérifié que dans chaque lot de cobayes la distribution des valeurs individuelles de la toxicité et de la durée de la perfusion suivait la loi normale. Nous avons calculé pour chaque série de n cobayes l'écart type σ_M de la moyenne m donnée par la formule suivante :

$$\sigma_M = \sqrt{\frac{\Sigma\,(x-m)^2}{n\,(n-1)}}$$

L'expression $\Sigma\,(x-m)^2$ placée sous le radical dite somme des carrés a été calculée de la manière habituelle suivante :

$$\Sigma\,(x-m)^2 = \Sigma\,x^2 - \frac{T^2}{n} \quad \text{où} \quad T = \Sigma\,x$$

A partir de σ_M on peut calculer les limites de confiance qui font intervenir σ_M et la valeur du t trouvée dans la table de Student [1] pour un nombre n d'animaux et au seuil de probabilité choisi, soit ici $P = 0{,}05$. Ces limites sont données par l'expression $m \pm \sigma t_M$.

Ces limites ainsi calculées ont la signification suivante : dans ces conditions, il y a 95 p. 100 de chances favorables pour affirmer que la vraie valeur de la toxicité ou la durée de la perfusion, en elle-même inconnue, soit comprise entre ces limites (Tableau I).

(1) Voir Statistical Table de R. A. Fisher et F. Yates, Oliver and Boyd, London 1953

TABLEAU I

Influence de différents sels de potassium sur la toxicité de la digitaline

Nombre d'animaux	Solutions perfusées	A. Toxicité de la digitaline en mg/kg.			B. Durée de la perfusion (en min.)		
		Moyenne	σ_M	Limites de confiance à P = 0,05	Moyenne	σ_M	Limites de confiance à P = 0,05
n							
20	Digitaline seule (2 séries cumulées)	1,60	± 0,0265	± 0,055	14,17	± 0,008	± 0,21
10	Dig. + ClK 0,10 g. p. 100	1,67	± 0,049	± 0,11	14,85	± 0,40	± 0,90
10	Dig. + ClK 0,21 g. p. 100	2,02	± 0,065	± 0,15	18,00	± 0,50	± 1,32
10	Dig. + ClK 0,25 g. p. 100	2,02	± 0,063	± 0,14	18,10	± 0,54	± 1,16
10	Dig. + ClK 0,30 g. p. 100	2,02	± 0,063	± 0,14	19,00	± 0,45	± 1,02
10	Dig. + ClK 0,35 g. p. 100	2,02	± 0,087	± 0,20	17,85	± 0,75	± 1,71
10	Dig. + ClK 0,40 g. p. 100	1,72	± 0,093	± 0,21	15,15	± 0,85	± 1,92
5	Dig. + ClK 0,60 g. p. 100	1,47	± 0,21	± 0,67	12,60	± 0,66	± 1,82
10	Dig. + Phosphate monopotassique à 0,615 g. p. 100 (K^+ = 0,157 g. p. 100)	1,78	± 0,071	± 0,61	16,10	± 0,66	± 1,48
10	Dig. + Phosphate bipotassique à 0,382 g. p. 100 (K^+ = 0,157 g. p. 100)	2,10	± 0,062	± 0,14	18,60	± 0,55	± 1,24
10	Dig. + Citrate de K à 0,142 g. p. 100 (K^+ = 0,051 g. p. 100)	2,35	± 0,050	± 0,13	21,25	± 0,71	± 1,50
10	Dig. + Citrate de K à 0,30 g. p. 100 (K^+ = 0,110 g. p. 100)	2,58	± 0,17	± 0,38	23,03	± 0,51	± 1,16
5	Dig. + Citrate de K à 0,425 g. p. 100 (K^+ = 0,156 g. p. 100)	1,90	± 0,043	± 0,12	15,86	± 1,61	± 4,45

TABLEAU I. *(Suite)*

Influence de différents sels de potassium sur la toxicité de la digitaline

10	Dig. + Phosphate bipotassique à 0,382 g. p. 100 (K [illegible] 0,15[illegible] g. p. 100)	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
10	Dig. + Citrate de K à 0,11[illegible] g. p. 100 (K⁺ = 0,051 g. p. 100)	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
10	Dig. + Citrate de K à 0,16 g. p. 100 (K⁺ = 0,116 g. p. 100)	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
5	Dig. + Citrate de K à 0,12[illegible] g. p. 100 (K⁺ = 0,156 g. p. 100)	1,00	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

TABLEAU I *(Suite)*

Influence de différents sels de potassium sur la toxicité de la digitaline

Nombre d'animaux n	Solutions perfusées	A. Toxicité de la digitaline en millig./kg. Moyenne	σ_M	Limites de confiance à P = 0,05	B. Durée de la perfusion (en min.) Moyenne	σ_M	Limites de confiance à P = 0,05
10	Dig. + fumarate de K (K^+ = 0,110 g. p. 100)	1,79	0,104	± 0,24	16,05	0,90	± 2,03
10	Dig. + fumarate de K (K^+ = 0,157 g. p. 100)	1,93	0,04	± 0,091	17,23	0,36	± 0,81
10	Dig. + Maléate de K (K^+ = 0,110 g. p. 100)	2,18	0,062	± 0,14	18,85	0,52	± 1,16
10	Dig. + Maléate de K (K^+ = 0,157 g. p. 100)	1,72	0,048	± 0,11	15,2	0,42	± 0,94
10	Dig. + Oxalate de K à 0,37 g. p. 100 (K^+ = 0,157 g. p. 100)	2,08	0,122	± 0,28	18,4	0,81	± 1,84
10	Dig. + Complexon II à 0,21 g. p. 100 (K^+ = 0,110 g. p. 100)	2,24	0,055	± 0,12	20,05	0,48	± 1,08
	A titre de comparaison nous avons étudié les deux séries suivantes avec le Citrate de Sodium						
10	Dig. + Citrate de Na à 0,131 g. p. 100	1,77	0,041	± 0,93	15,80	0,38	± 0,86
10	Dig. + Citrate de Na à 0,30 g. p. 100	1,84	0,074	± 0,17	16,25	0,60	± 1,36
10	Dig. + Citrate de Na à 0,393 g. p. 100	1,81	0,069	± 0,16	16,2	0,67	± 1,51

2. Pour tester le degré de signification de la différence entre les 2 moyennes des lots à comparer deux à deux (cas d'échantillons indépendants) nous avons déterminé la valeur du t de Student-Fisher.

Cette valeur de t est calculée au moyen de la formule :

$$t = \frac{m_1 - m_2}{\sqrt{\frac{\Sigma (x_1 - m_1)^2 + \Sigma (x_2 - m_2)^2}{n_1 + n_2 - 2} \left(\frac{1}{n_1} + \frac{1}{n_2}\right)}}$$

où m_1 et m_2 sont les moyennes à comparer, n_1 et n_2 le nombre d'animaux dans chaque lot et $\Sigma_1 (x_1 - m_1)^2$ qui représente la somme des carrés du lot 1 et $\Sigma_2 (x_2 - m_2)^2$ celle du lot 2.

Connaissant la valeur de t calculé, on peut la comparer à la valeur t trouvée dans la table pour le nombre $n_1 + n_2 - 2$ degrés de liberté correspondant. Il en résulte qu'on peut situer le niveau de probabilité P correspondant à la comparaison qui est indiquée dans le tableau II avec sa signification.

Interprétation des Résultats

a) L'addition de *chlorure* de potassium (Fig. 1), si sa concentration est suffisante, diminue la toxicité de la digitaline.

Si la concentration est trop faible (0,10 g p. 100) cette diminution n'est pas notable : la dose moyenne mortelle par kg passe de 1,60 ± 0,055 pour la digitaline seule à 1,67 ± 0,11, la différence n'est pas significative (P = 0,2).

L'addition de ClK à 0,21 g p. 100 entraîne une différence très significative par rapport à la digitaline seule. La D.M.M. passe de 1,60 ± 0,055 à 2,02 ± 0,15 (P = 0,001) et par rapport à la digitaline additionnée de ClK à 0,10 g p. 100, P = 0,001 également.

Il semble s'agir là de la dose minimale efficace de ClK, très proche aussi de la dose optimale car de plus fortes concentrations (0,25 g — 0,30 g — 0,35 g) n'améliorent pas l'effet obtenu [voir sur la fig. 1 la courbe ajustée à la distribution (+ + + +)] : l'action optimale correspond à 0,25 0,28 g p. 100 de ClK. Avec des doses supérieures de ClK (0,40 et 0,60), la dose minimale mortelle du mélange redevient voisine de celle de la digitaline ou même tombe au-dessous de celle-ci, probablement du fait de la toxicité propre du potassium.

b) L'activité du *phosphate* monopotassique est nette; la D.M.M. passe de 1,60 ± 0,055 pour la digitaline seule à 1,78 ± 0,61 pour le

mélange digitaline + phosphate monopotassique à 0,61 g p. 100 (K^+ = 0,157 g p. 100) pour un seuil de probabilité $P < 0,001$.

L'action du phosphate bipotassique est moins nette : la D.M.M.

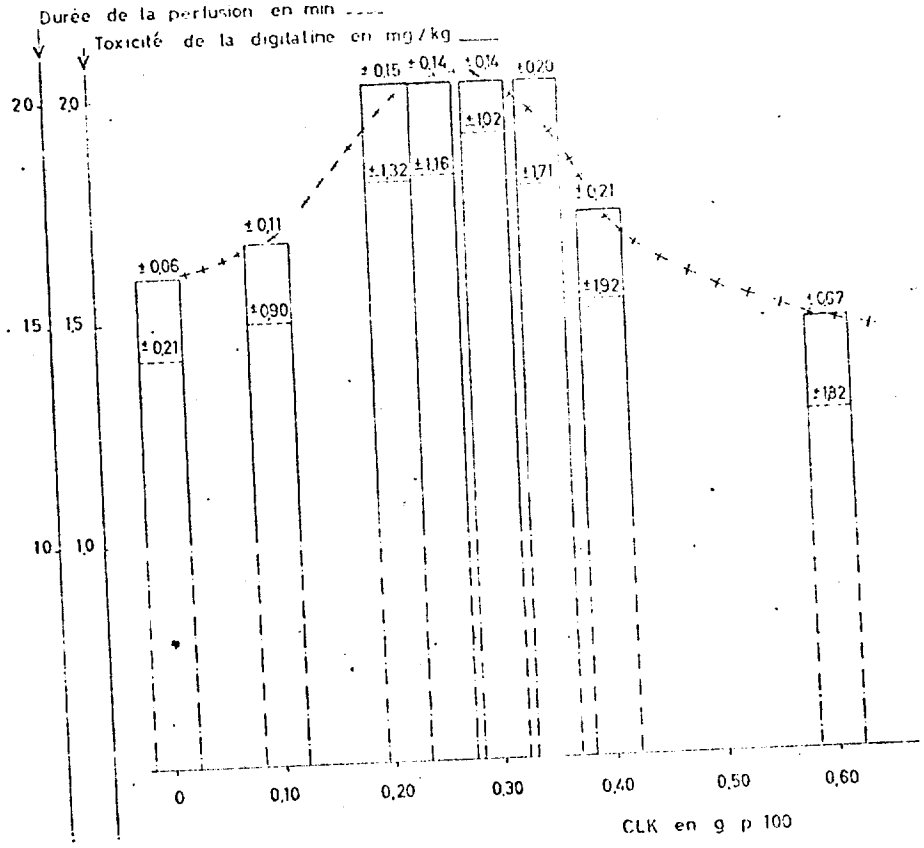

FIG. 1

Toxicité de la digitaline (en milligrammes par kg) seule et en présence de chlorure de potassium à concentrations croissantes. La durée de la perfusion est figurée en traits rompus (- - -). Nous avons indiqué au-dessus de chaque rectangle constituant l'histogramme, les limites de confiance de la moyenne de chaque échantillon indiquées au tableau I. Le nombre d'animaux dans chaque série est indiqué au tableau I. Afin de réduire l'étendue de la figure dans le sens vertical nous l'avons coupée horizontalement.

Nous avons tenté de tracer une courbe ajustée à la distribution (| - - - |); On peut voir que l'action antitoxique optimale du ClK vis-à-vis de la digitaline correspond à des concentrations de ClK situées au voisinage de 0,25 à 0,28 g. p. 100.

passe de 1,60 ± 0,055 pour la digitaline seule à 2,10 ± 0,14 après addition à la digitaline de phosphate bipotassique pour un seuil de probabilité $P < 0,02$.

L'action favorable de l'ion K, dans certaines limites, peut-elle être attribuée à son antagonisme à l'égard de l'ion calcium ? Dans ce cas,

l'inactivation de ce dernier devrait favoriser encore davantage l'action détoxiquante du potassium à l'égard de la digitaline.

c) *L'oxalate de potassium* (1) (K^+ = 42,4 p. 100) en solution à 0,37 g p. 100, qui en fait la précipitation, ne protège pas plus (D.M.M. = 2,08 ± 0,28) que le chlorure de potassium à la dose optimale de 0,21 g p. 100

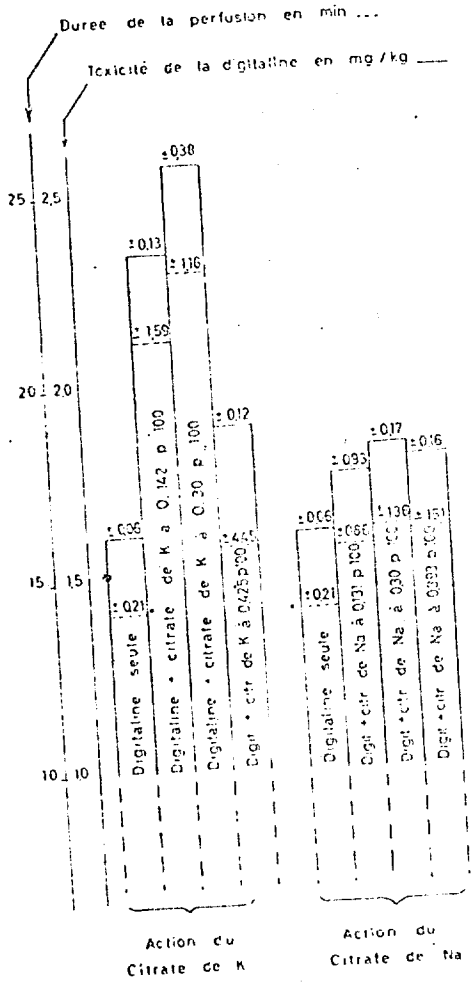

FIG. 2

Action comparée des citrates de K et de Na sur la toxicité de la digitaline indiquée comme pour les fig. 1 par la toxicité en mg/kg. et le volume de la perfusion toxique utilisé. Les indications générales sont identiques à celles qui ont été données pour la fig. 1. Il ressort nettement que si l'action antitoxique propre à l'ion citrate apparaît pour le citrate de Na, l'ion potassium renforce considérablement cet effet.

(1) La toxicité moyenne propre de cet oxalate en solution à 0,37 g. p. 100 est de 33,28 ± 2,15 cm³ p. kg. Durée de perfusion 29,66 ± 1,85 minutes (moyenne ± $\sigma/\sqrt{n}$ calculée sur une série de 10 cobayes).

(D.M.M. = 2,02 ± 0,15) sans doute en raison de la toxicité propre de l'ion oxalate pour le cœur (R. HAZARD, L. WURMSER et J. CHEYMOL, 4).

d) Le *citrate de potassium* (FIG. 2) diminue plus la toxicité de la digitaline que le chlorure pour des sels contenant la même quantité d'élément K^+ (0,110 g p. 100). On constate une différence nette entre la D.M.M. de la digitaline seule (1,60 ± 0,055) et celle du mélange digitaline + citrate de potassium à 0,30 g p. 100 (2,58 ± 0,38) au seuil de probabilité P = 0,001; ceci est valable également pour la durée de perfusion.

Cette concentration de citrate de potassium à 0,30 g p. 100 semble voisine de la concentration optimale, car si l'on porte la dose de citrate de K à 0,425 g p. 100, la D.M.M. s'abaisse à 1,90 ± 0,12, écart significatif de la D.M.M. obtenue avec le citrate de K à 0,30 au seuil de probabilité P = 0,05.

Après addition de *citrate de sodium* (FIG. 2) à 0,30 g p. 100, la D.M.M. de la digitaline passe de 1,60 ± 0,055 à 1,84 ± 0,17; il semble que l'ion citrate exerce une action propre qui augmente la dose mortelle de digitaline; en effet : 1) à poids égal de K^+ (0,110 g p. 100) le citrate de potassium augmente la dose mortelle par rapport au ClK; 2) le citrate de sodium augmente à lui seul, indépendamment du potassium, la dose mortelle.

Enfin entre le citrate de sodium (D.M.M. = 1,84 ± 0,17) et le citrate de potassium (D.M.M. = 2,58 ± 0,38) existe une différence significative, due évidemment à la présence de K^+, au seuil de probabilité P = 0,01. Le potassium exerce bien dans la molécule du citrate de potassium une action spécifique.

e) L'addition de *fumarate de potassium* (1) (FIG. 3) à diverses concentrations (K^+ = 0,110 g et 0,157 g p. 100) ne modifie pas d'une façon valable la toxicité de la digitaline seule.

f) Le *maléate de potassium* (FIG. 3) à K^+ = 0,110 g p. 100 diminue très nettement la toxicité de la digitaline : la D.M.M. de la digitaline passe de 1,60 ± 0,055 à 2,18 ± 0,14 au seuil de probabilité P = 0,001. Cette concentration de maléate de K semble voisine de la dose optimale, car si on l'élève, la D.M.M. s'abaisse à 1,72 ± 0,11 (K^+ = 0,157 g p. 100).

(1) La toxicité moyenne du fumarate de K pour une solution à 0,5 g. p. 100 en K est de 0,214 ± 0,044 cm³ par kg. Durée de perfusion 37,9 ± 0,95 minutes. (moyenne ± $\sigma/\sqrt{n}$ calculé sur 9 cobayes).

De même pour le maléate à 0,5 g. p. 100 en K la toxicité moyenne est de 0,182 ± 0,016 cm³ par kg. Durée de perfusion 32,70 ± 0,25 minutes (moyenne ± $\sigma/\sqrt{n}$ calculé sur 10 cobayes).

g) L'emploi d'un sel de K du *complexon II* (1) à 0,21 p. 100 (K^+ = 0,110 p. 100) permet d'élever la D.M.M. de la digitaline de 1,60 ± 0,055 à 2,24 mg/kg ± 0,12, avec une différence très forte (P = 0,001).

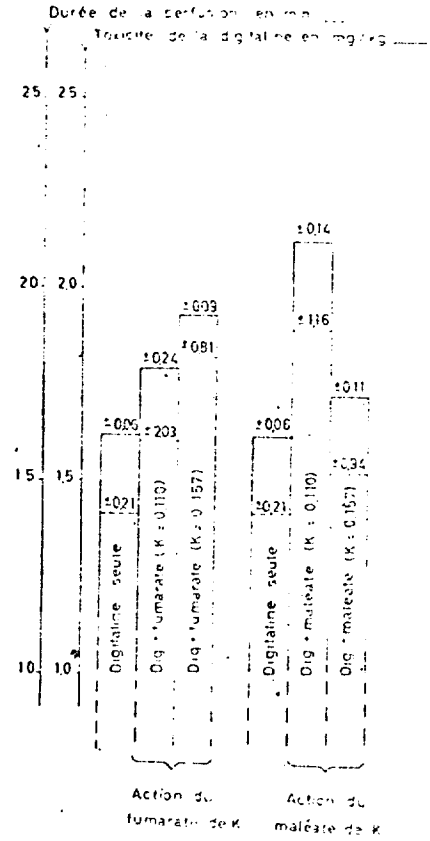

FIG. 3

Action du fumarate de K et du maléate de K à diverses concentrations sur la toxicité de la digitaline. Le lot témoin digitaline seule est situé à gauche de chaque histogramme. Comme pour les fig. 1 et 2 nous avons amputé la figure dans le sens vertical pour en réduire les dimensions sans en réduire l'échelle. Les chiffres placés au-dessus de chaque rectangle indiquent les limites de confiance de la moyenne (voir TABL. 1).

On peut voir que le fumarate et le maléate de K se comportent de manière différente : le maléate a une action optimale pour une marge de concentrations étroite; pour le lot fumarate de K nous n'avons peut-être pas encore atteint l'effet optimal.

(1) Les toxicités moyennes du complexon II acide pour des solutions correspondant à K^+ 0,21 g. p. 100 et à K^+ 0,30 g. p. 100 ont été respectivement pour la première de 49,26 ± 0,20 cm³ par kg. (Durée de perfusion 22,90 ± 2,30 minutes) (moyenne ± $\sigma/\sqrt{n}$ calculé sur 10 cobayes); pour la seconde de 25,15 ± 2,81 cm³ par kg. (Durée de perfusion 32,70 ± 0,25 minutes) (moyenne ± $\sigma/\sqrt{n}$ calculé sur 12 cobayes).

TABLEAU II

Comparaisons entre les lots; degré de signification de la différence entre les moyennes de chaque lot

TABLEAU II *(suite)*

Comparaisons entre les lots; degré de signification de la différence entre les moyennes de chaque lot

degrés de liberté $n_1 + n_2 - 2$		A. Toxicité de la digitaline (10^{-4})			B. Durée de la perfusion (en min.)		
		t calculé	seuil de probabilité P	signification de la différence	*t* calculé	seuil de probabilité P	signification de la différence
	2. Digitaline et phosphate monopotassique (K = 157 mg. p. 100)						
18	Digitaline seule / Digit. + PO_4KH_2	6,08	< 0,001	très grande	2,81	0,02	grande
	3. Digitaline et phosphate bipotassique (K = 157 mg. p. 100)						
18	Digit. seule et / Digit. + PO_4K_2H	2,60	0,02	assez grande	7,48	0,001	grande
	4. Digitaline et Citrate de potassium						
18	Digit. seule / Digit. + Citrate de K à 0,142 g. p. 100	14,58	< 0,001	très grande	9,51	0,001	très grande
18	Digit. + Citrate de K à 0,142 g. p. 100 / Digit. + Citrate de K à 0,30 g. p. 100	1,27	≥ 0,2	faible	2,05	≥ 0,05	très grande
18	Digit. + Citrate de K à 0,30 g. p. 100 / Digit. + Citrate de K à 0,42 g. p. 100	2,74	< 0,02	grande	6,73	0,001	très grande

TABLEAU II *(Suite)*

Comparaisons entre les lots; degré de signification de la différence entre les moyennes de chaque lot

	A. Toxicité de la digitaline (10^{-4})	B. Durée de la perfusion (en min.)

TABLEAU II (*Suite*)

Comparaisons entre les lots; degré de signification de la différence entre les moyennes de chaque lot

degrés de liberté $n_1 + n_2 - 2$		A. Toxicité de la digitaline (10^{-4})			B. Durée de la perfusion (en min.)		
		t calculé	seuil de probabilité P	signification de la différence	*t* calculé	seuil de probabilité P	signification de la différence
	5. Digitaline et Citrate de Sodium (à comparer avec 4.)						
18	Digit. seule et Digit. + Citrate de Na à 0,131 g.p. 100	3,69	0,01 — 0,001	très grande	3,65	0,01 — 0,001	très grande
18	Digit. + Citrate de Na à 0,131 g.p. 100 / Digit. + Citrate de Na à 0,30 g.p. 100	1,09	⩽ 0,3	faible	0,49	> 0,6	nulle
18	Digit. + Citrate de Na à 0,30 g.p. 100 / Digit. + Citrate de Na à 0,39 g.p. 100	0,31	—	nulle	0,054	—	nulle
	5. bis. Comparaisons entre citrates de K et de Na						
18	Digit + Citrate de K à 0,30 g.p. 100 / Digit. + Citrate de Na à 0,30 g.p. 100	3,97	0,01	grande	8,13	0,001	très grande
13	Digit. + Citrate de Na à 0,39 g.p. 100 / Digit. + Citrate de K à 0,42 g.p. 100	0,91	0,4	nulle	0,82	0,4	nulle
	6. Digitaline et fumarate de potassium						
18	Digitaline seule / Digit. + fumarate de K (K^+ = 0,110 g. p. 100)	1,90	0,1	faible	2,13	0,05	sensible
18	Digit. + fumarate de K (K^+ = 0,110 g. p. 100) / Digit. + fumarate de K (K^+ = 0,157 g. p. 100)	1,27	0,2	nulle	1,22	0,2	nulle

TABLEAU II *(Suite)*

Comparaisons entre les lots; degré de signification de la différence entre les moyennes de chaque lot

degrés de liberté $n_1 + n_2 - 2$		A. Toxicité de la digitaline (10^{-4})			B. Durée de la perfusion (en min.)		
		t calculé	seuil de probabilité P	signification de la différence	*t* calculé	seuil de probabilité P	signification de la différence
	7. Digitaline et maléate de potassium						
18	Digitaline seule / Digit. + maléate de K (K^+ = 0,110 g. p. 100)	7,65	0,001	très forte	8,28	0,001	très forte
18	Digit. + maléate de K (K^+ = 0,110 g. p. 100) / Digit. + maléate de K (K^+ = 0,157 g. p. 100)	5,00	0,001	très forte	5,53	0,001	très forte
	8. Digitaline et Oxalate de potassium						
18	Digitaline seule / Digit. + Oxalate de K (K^+ = 0,157 g. p. 100)	3,96	0,01 > P > 0,001	forte	5,06	0,001	très forte
	9. Digitaline et Complexon II acide						
18	Digitaline seule / Digit. + Complexon à 0,210 g. p. 100	10,27	0,001	très forte	11,1	0,001	très forte

TABLEAU II

Comparaisons entre les lots; degré de signification de la différence entre les moyennes de chaque lot

degrés de liberté $n_1 + n_2 - 2$	A. Toxicité de la digitaline (10^{-4})				B. Durée de la perfusion (en min.)		
		t calculé	seuil de probabilité P	signification de la différence	t calculé	seuil de probabilité P	signification de la différence
	1. Digitaline et Chlorure de potassium						
18	Digitaline seule Digitaline + ClK à 0,10 p. 100	1,27	0,2	nulle	1,66	$0,2 > P \geqslant 0,1$	nulle
18	Digit. + ClK 0,10 p. 100 Digit. + ClK 0,21 p. 100	4,37	0,001	très grande	4,38	0,001	très grande
18	Digit. + ClK 0,21 p. 100 Digit. + ClK 0,25 p. 100			nulle	0,129	0,9	nulle
18	Digit. + ClK 0,25 p. 100 Digit. + ClK 0,30 p. 100	0,18	0,9	nulle	1,31	0,2	nulle
18	Digit. + ClK 0,30 p. 100 Digit. + ClK 0,35 p. 100	1,08	0,3	nulle	1,30	0,2	nulle
18	Digit. + ClK 0,35 p. 100 Digit. + ClK 0,40 p. 100	2,34	0,05	sensible	2,38	0,05	sensible
13	Digit. + ClK 0,40 p. 100 Digit. + ClK 0,60 p. 100	2,184	0,05	sensible	2,83	0,02	assez forte

18	Digitaline seule	10,27	0,001	très forte	11,1	0,001	très forte
	Digit. + Complexon à 0,210 g. p. 100						
	9. Digitaline et Complexon II acide						

Conclusions

Les différents sels de potassium utilisés protègent tous le cœur du cobaye contre les effets toxiques de la digitaline.

Leur activité varie suivant l'anion du sel; elle passe par une valeur maximale puis s'abaisse au-delà d'une concentration optimale.

Le plus efficace est le citrate de potassium, qui fait passer la dose mortelle moyenne du glucoside de 1,60 à 2,58 mg/kg.

Dans l'ordre décroissant de la protection et d'après les meilleurs résultats enregistrés viennent successivement : le sel de potassium du complexon II (2,24), le maléate (2,18), le phosphate (2,10), l'oxalate (2,08), le chlorure (2,02), le fumarate (1,93). L'inactivation du calcium joue un rôle, mais elle est réalisée sans doute par des processus assez divers mal définis en milieu complexe et il ne semble pas possible de préciser ceux qui réalisent les conditions les meilleures.

Malgré la rigueur mathématique qui a présidé au calcul des chiffres présentés, il est évident que ceux-ci n'ont rien d'absolu et qu'ils mériteraient d'être complétés par une expérimentation beaucoup plus large encore. Mais même s'ils ne représentent qu'une approximation, ils peuvent cependant permettre d'attribuer à l'ion potassium une action détoxiquante relative à l'égard de la digitaline.

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Myocardial Injury Associated with Asystole Induced with Potassium Citrate *

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SUCCESSFUL total body perfusion has brought recognition of new problems which could not be anticipated fully before the use of this technic for intracardiac surgery. Several of these problems such as excessive coronary flow and the technical difficulty of suture and prosthesis placement in the actively beating, bypassed heart suggest the value of elective cardiac arrest. Use of potassium citrate solution as suggested by Melrose for arrest of the heart was tried in our laboratory, but the results were not entirely satisfactory. The present report is concerned especially with untoward effects encountered when the Melrose technic was used with our method of extracorporeal oxygenation and circulation.

Method

The dogs used in these experiments varied considerably in size and included several small puppies. No animals with anemia or respiratory infection were used but aside from this they were unselected mongrels that had been prepared with a vermifuge. Sodium pentobarbital was the anesthetic agent used and ventilation was through a large tracheal tube by a mechanical ventilator with a suction phase in its cycle. Peripheral vessels were cannulated so that pressure was measured in the superior and inferior vena cava and the abdominal aorta. The right thorax was opened through the fourth intercostal space and in most instances the sternum was transected to permit a short extension of the incision in the same interspace to the left of the midline. The cannulation of the cavae for the drainage of venous blood into the heart-lung machine was done through the wall of the right atrium. The cannula for return flow from the extracorporeal system was inserted into the right common femoral artery and directed toward the heart.

The system for complete drainage of blood from the left side of the heart included two parts as illustrated in Figure 1. A large stainless steel cannula was passed through the appendage into the left atrium. This was attached to a suction line which carried the blood into the coronary pumps of the heart-lung machine. Another steel cannula was inserted into the left ventricle through a stab at the apex.[1] This was connected to a line which also carried the blood to the coronary pumps but there was no suction in this system and it drained only by gravity.†

* Submitted for publication March 24, 1958.

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This work was supported by grants from the United States Public Health Service (H-2427) and the American Heart Association.

† It is recognized that a septal defect exists in many conditions for which open heart surgery is performed at present. Right atriotomy or ventriculotomy results, therefore, in some degree of venting of the left side chambers. We do not believe that drainage of the left heart through a

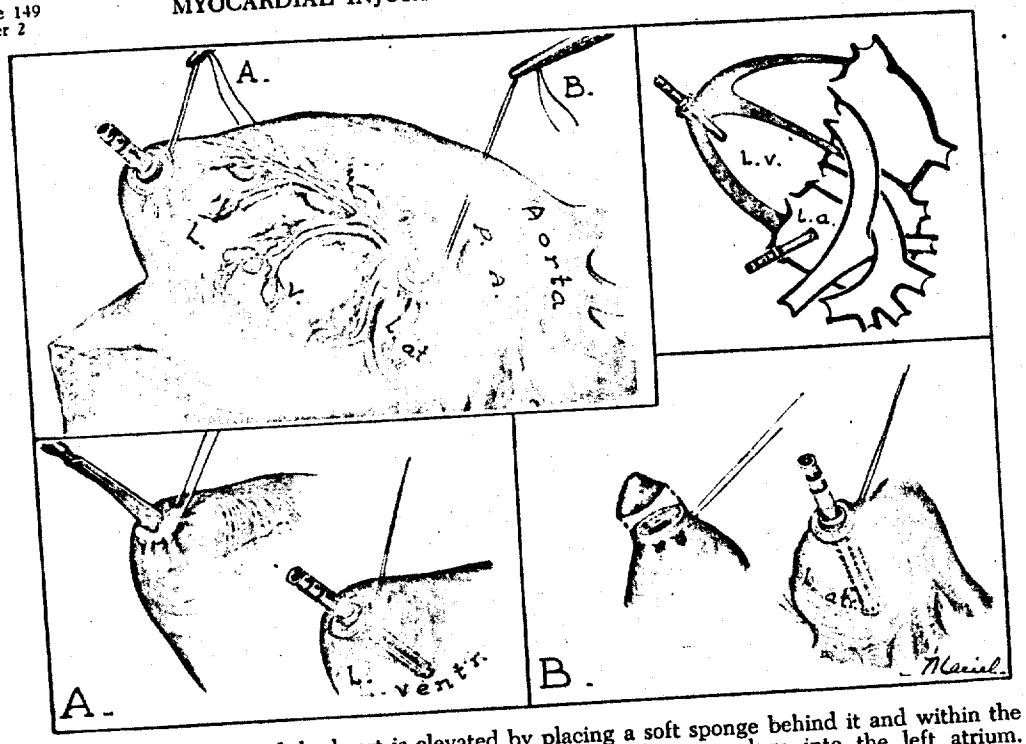

FIG. 1. The apex of the heart is elevated by placing a soft sponge behind it and within the pericardium. A large steel cannula is passed through the appendage into the left atrium. Another cannula is passed through a stab wound into the left ventricle.

The perfusion rate was predetermined according to calculation by L. C. Clark.[2] As a rule the flow through the oxygenator pump was never less than this arbitrary value and frequently it exceeded the calculated figure. The pressure in the superior and inferior caval systems as well as the arterial pressure were considered also in determining the rate of the perfusion.

Other monitoring instruments supplied information of greater importance perhaps and these were the principal determinants of flow rate during the course of each perfusion. The partial pressure of oxygen in the arterial blood was measured polarigraphically with a platinum electrode.[3] The pH of the "arterialized" blood was recorded continuously with a general purpose glass electrode and a fiber-type reference electrode with a Beckman pH meter.[4] The temperature of the blood was kept normal by a small heating unit which warmed the blood as it entered the extracorporeal circuit.

Experiments in the first part of this study are divided into three groups. The first group consisting of 15 animals, three of which were pups, were subjected to total cardiac bypass for 30 minutes. The cardiac rhythm and the flow in coronary vessels were not altered during the experiment. The system for complete drainage of the left heart was employed and in all other respects the perfusions were conducted as our perfusions are at present in clinical practice. These experiments, therefore, are the control series.

The second group included 17 animals in which the hearts were arrested with potas-

septal defect and right cardiotomy accomplishes the same effect as the cannulas in the left atrium and ventricle. However, further comparison of these two methods is not pertinent to this experimental study.

TABLE 1

	Av. Flow Rate cc./Kg./min.	Av. Mean Arterial Pressure mm. Hg	Av. Arterial pH	Deaths
Perfusions in Sinus Rhythm				
15 with complete drainage left atrium	62 (53–81)	66 (40–90)	7.43 (7.35–7.50)	0
Perfusions in Arrest by K Citrate				
17 with incomplete drainage left atrium	61 (52–71)	92 (70–100)	7.46 (7.40–7.52)	8
7 with complete drainage left atrium	89 (65–116)	95 (90–120)	7.43 (7.40–7.47)	1

Range is given in figures in parentheses.

sium citrate. The salt was diluted with arterial blood in the way Melrose described, and his technic for injection was duplicated exactly. In these experiments, however, there was incomplete drainage of blood from the chambers of the left side of the heart during the 30-minute interval of arrest and total extracardiac perfusion. In several instances blood was released from the left atrium during measurement of pressure in that chamber. The volume that escaped from the left heart was relatively small, however, and no doubt was less than the amount entering via the bronchial arteries and other collateral vessels.

The third group consisted of seven animals in which the hearts were arrested by potassium citrate with an exact duplication of the Melrose technic. This group, however, had complete drainage of blood from the left atrium and ventricle during the 30-minute period of arrest and perfusion of the remainder of the body.

Results and Discussion

The perfusions carried out with the heart in sinus rhythm were uniformly successful. Ventricular fibrillation complicated three of the experiments but it was easily eliminated by electroshock, and all of the animals survived. The average flow rate, arterial pressure, and arterial pH are given in Table 1. The volume of blood that drained from the left atrium during the interval of total body perfusion was measured several times each during seven of the experiments. In most instances it varied between 30 and 90 cc. per minute. In the experimental conditions of this group this amount of blood was derived from at least two sources, the coronary and the bronchial arterial system.

The perfusions performed during arrest of the heart with potassium citrate and without drainage of the left atrium comprised a group with results that were strikingly different. After injections of the potassium salt and the production of asystole we observed that five hearts developed a true rigor of the left ventricle. This localized change in tone could not be altered by massage or flushing the coronary arteries with blood, and the complication was uniformly fatal.[5] Altogether, eight animals in the group died, and in every instance they developed ventricular fibrillation after the aorta was opened following the 30-minute period of arrest and interruption of coronary circulation. Table 1 lists additional data on the flow rate, arterial pressure, and arterial pH maintained during these perfusions. As

Arterial pH	Deaths
7.43 (7.35–7.50)	0
7.46 (7.40–7.52)	8
7.43 (7.40–7.47)	1

rate, arterial pres-
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TABLE 2

	Heart Arrested During Perfusion										
				Asystole by Anoxia				Asystole by K Citrate (Left Chambers Drained)			
Heart in Sinus Rhythm During Perfusion				Sacrificed Immediately		Sacrificed After 48 Hrs.		Sacrificed After 48 Hrs.			
Exp. No.	Sacrificed After 48 Hrs. CR-14	CR-15	CR-16	231	230	229	228	232	227	226	224
Focal myocardial necrosis	0	+1	±	0	0	0	±	+++	+++	++++	++++[2]
Mineralization of necrotic muscle	0	0	0	0	0	0	0	0	+++	+++	+++
Fatty degeneration of myocardium	0	+	++	0	0	++++	++	+++	+++	+++	0
Focal hemorrhage	0	0	0	+	+	+	0	+	+	0	++

1. Foci of hyaline degeneration in a papillary muscle.
2. Subendocardial infarction.

nentioned earlier, a small amount of blood was drained from the left atrium in eight animals in this group. The single objective in this maneuver was to measure the pressure and to prevent engorgement of the pulmonary veins.

Perfusions done during potassium citrate arrest but with complete drainage of the left atrium gave significantly different results in a group of seven animals. The peculiar tonic contraction of the left ventricle was not observed a single time. Ventricular fibrillation complicated only two of the experiments and the single death in the group was caused by an accidental aortic tear which could not be repaired; additional information on flow rate, arterial pressure, and arterial pH is given in Table 1. The volume of blood collected from the left atrium during perfusion and while the aorta was closed above the coronary orifices was a measurement of flow through the bronchial arteries. There was a wide variation between experiments; in most animals it was from ten to 20 cc. per minute. The lowest measurement recorded was five and the highest 38 cc. per minute.

The striking difference in mortality rate between the first and third groups on one hand and the second group on the other suggests that drainage of blood from the left atrium has a very beneficial effect upon cardiac function during this type of perfusion. We believe that part of the mechanism for this is the prevention of elevated pressure in the chambers of the left heart—pressure which may result not only in overstretched myocardial fibers but also, when transmitted along pulmonary veins, may cause harmful engorgement of the lungs. Drainage of the left atrium may provide an additional benefit when the Melrose technic is used for asystole. Our measurements have shown that a relatively high concentration of potassium is found in blood from the pulmonary veins and left atrium following the intra-aortic injection of Melrose's solution and we believe that this may be harmful to the myocardium if it is allowed to collect in the left chambers for 30 minutes.[6]

In addition to observations on the immediate reaction of the heart and the rate of survival in the different experimental groups, microscopic examination was made on blocks from the ventricles of three additional groups of dogs. The results of this

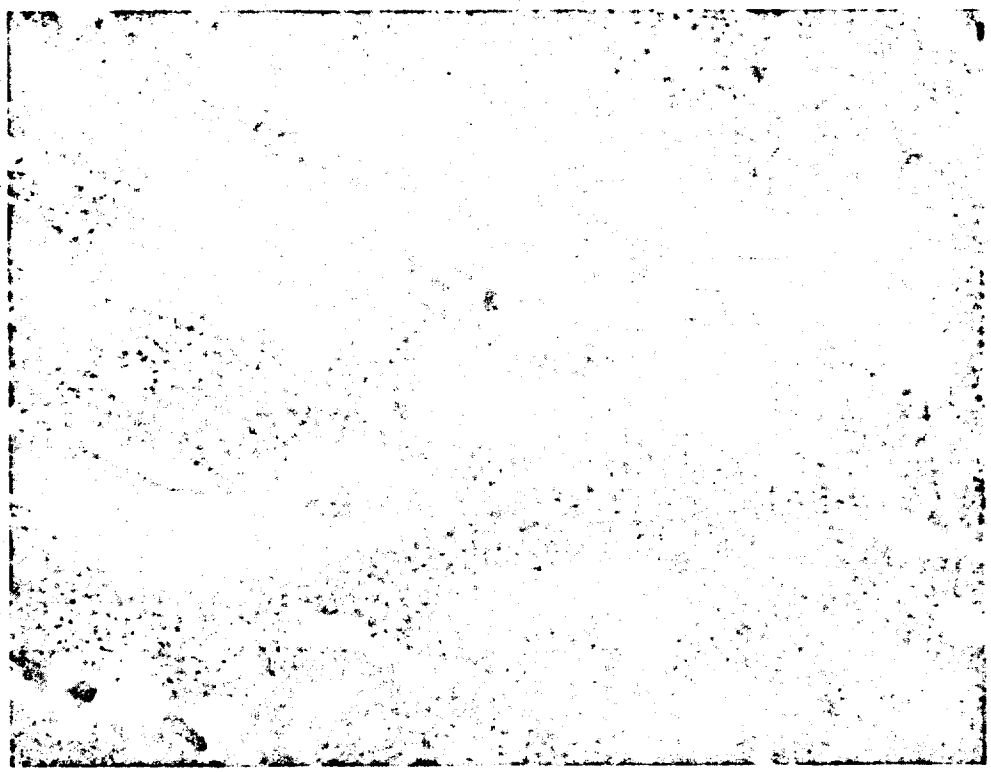

FIG. 2. Dog 229. Fine droplets of sudanophilic material in myocardial fibers. (Sudan IV ×650.)

part of the study have been abbreviated and listed in Table 2.

The first group consisted of three dogs which served as controls for the ones to follow. Their hearts were examined pathologically 48 hours after total bypass. Coronary flow was not altered in any way and no arresting agent was employed during these perfusions. The myocardium of one animal was entirely normal. The second dog had a rare focus of necrobiotic fibers in a papillary muscle of the left ventricle as well as occasional foci of fatty degeneration in the myocardium of both ventricles. The third specimen had rare foci with hyaline appearing muscle fibers but no definite necrobiosis. There were fatty changes in the myocardium, however, which were generalized in distribution with focal areas of accentuation.

The second group subjected to pathological examination of the heart were ones in which arrest was produced by anoxia resulting from 30 minutes of occlusion of the aorta just above the coronary ostia. Two hearts were removed for examination immediately after the perfusion. The only lesions found in these were focal myocardial hemorrhage. Two other dogs subjected to the same type of perfusion and myocardial anoxia withstood the experiment well and 24 hours later were in good general condition. They were sacrificed 48 hours after the experiment and one heart showed diffuse fatty degeneration, but no necrobiosis (Fig. 2). The other had a rare focus suggestive of necrotic muscle fibers and scattered areas of fatty degeneration. In this latter specimen there was a mantle of fatty degeneration beneath the epicardium, a lesion possibly attributable to a thermal effect of operating lights upon the exposed cardiac surface.

The third group studied histologically in-

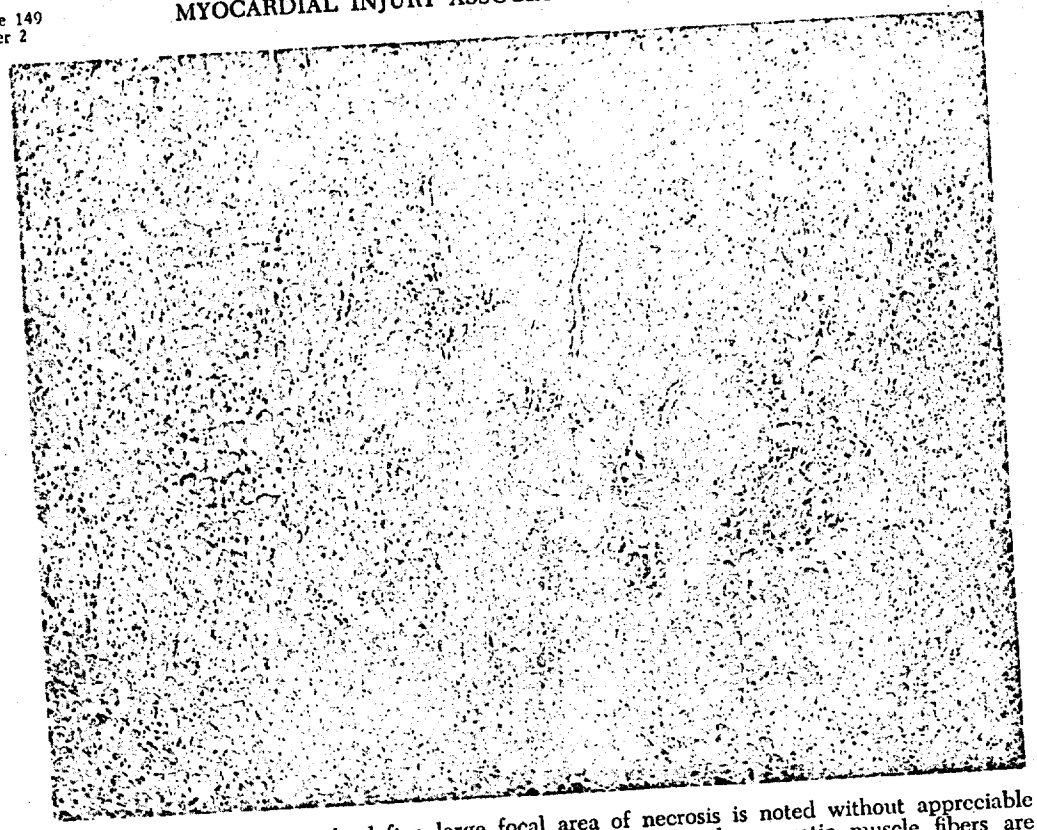

FIG. 3. Dog 226. On the left a large focal area of necrosis is noted without appreciable evidence of inflammation. In the smaller foci to the right the necrotic muscle fibers are mineralized. (Hematoxylin eosin ×90.)

cluded four dogs arrested with potassium citrate and with the left heart drained by the technic described. The animals were allowed to recover from the perfusion and then were sacrificed after 48 hours. Lesions of the myocardium were visible grossly in all of these specimens. These consisted of yellow-grey opaque streaks in the myocardium which were found to be friable. In one dog there was excessive necrosis in association with a cardiotomy wound in the right ventricle. Microscopically all four specimens showed extensive focal areas of myocardial necrosis in which the fibers were shrunken, opaque, and deeply eosinophilic. In three specimens there was mineralization of necrotic muscle fibers in many areas (Fig. 3). The pattern of necrosis was random in both ventricles but often tended to be about branches of the coronary arteries. One animal had a subendocardial infarct. Sudan IV preparations in three specimens demonstrated fine droplet fatty degeneration of a focal type, particularly disposed about the necrotic areas.

The pathologic studies indicate that with total perfusion by our method there is some myocardial damage when asystole is not induced. The fatty degeneration was of a mild degree, however, and frank necrobiosis was limited to rare foci. Asystole produced by potassium citrate was associated with a serious degree of myocardial necrosis and this developed in every instance despite use of a method for drainage of blood from the left heart during total perfusion. Thus the findings on pathologic study did not confirm the impression from experiments in perfusion and left heart drainage in which the main criterion was survival of the animal.

Summary

Arrest of the ventricles by injection of potassium citrate solution resulted in several complications not encountered when total body perfusion is carried out with the heart in sinus rhythm. An unusual rigor of the left ventricle was seen in many of the arrested hearts and irreversible ventricular fibrillation accounted for the high mortality rate in this group. A technic was developed for the complete drainage of blood from the left heart chambers and when this was used with Melrose's method for arrest, ventricular rigor and irreversible fibrillation did not develop in a single experiment.

Hearts from three groups of animals were examined pathologically 48 hours after total perfusion. The first group were three controls, perfused without arrest or alteration in the coronary circulation, and these showed some fatty degeneration. The hearts of four dogs subjected to anoxic arrest by aortic clamping were examined and found to show focal hemorrhages and fatty degeneration but no necrosis. Specimens from four animals arrested with potassium citrate and with complete drainage of the left chambers showed focal areas of necrosis in every instance.

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inuation in the size of the bone will result. A second intravenous injection is recommended in ten to 14 days.

Summary and Conclusions

Sodium iodide administered intravenously has been successfully used in the treatment of actinomycosis for a period of over four years.

Advantages of the intravenous use of sodium iodide in treating actinomycosis are: Treatment of this condition is too often taken care of by the layman through his knowledge of the use of potassium iodide. Laymen, after seeing the advantages of the use of sodium iodide in comparison with that of potassium iodide, are forced to employ a veterinarian because of its method of administration.

Sodium iodide treatment does not cause a loss of flesh in animals that are being fitted for beef; any slight decrease in lactation in dairy cows is but temporary; animals in which actinomycosis appears previous to shipment can be treated without any bad results or delay in shipment.

The intravenous administration of sodium iodide is productive of a higher percentage of recoveries and more certain results than with any other form of treatment; it is safe to use; only one treatment is necessary; the cost is negligible; iodism is not produced, and bone lesions react favorably to its use.

The intravenous use of sodium iodide usually causes abortion in pregnant animals.

In addition to the method of medicinal treatment, it returns to the profession a service that can be rendered only by a veterinarian.

Acknowledgment

I desire to express my appreciation to Dr. N. J. Miller, of Eaton, Colorado. Both of us started working on this at about the same time and Dr. Miller has coöperated in helping to make it successful.

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The International Live Stock Exposition will be held in Chicago, November 27-December 4.

THE EFFECTS OF VARIOUS SODIUM, POTASSIUM, AND CALCIUM SALTS ON RESPIRATION AND BLOOD-PRESSURE IN THE DOG*

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It has long been known that calcium, sodium and potassium ions in the blood and lymph are necessary for the proper functioning of certain body tissues. The compounds used in these studies were selected because of their importance from a clinical and metabolic standpoint. The experiments recorded in this paper were performed in conjunction with a series of studies on the effects of various fluorides and related compounds upon respiration and blood-pressure, the results of which have been or are in the process of being published.

Description of the Animals

All of the experiments were performed on dogs. Seven dogs were used, four males and three females. The smallest weighed 5.49 kilos and the largest 20 kilos. The exact ages of the dogs were not known, although they were all mature animals.

Methods and Apparatus

The experimental methods are those ordinarily used in pharmacological and physiological studies involving the use of a long-paper kymograph, mercury manometer, pneumograph, time marker and signal magnet. Three per cent pento-barbital-sodium (Abbott), administered either intraperitoneally or intrapleurally on the basis of 30 mg per kilo of body weight, is ordinarily employed for anesthesia. The general procedure in performing an experiment was to inject small quantities of the compound, after a preliminary tracing had been made. The preliminary tracing was assumed to be normal for the animal under anesthesia; thus the effects on the blood-pressure and respiration resulting from the injection of the salts could be evaluated. The dosage of the salt was increased gradually, until a significant change was produced on respiration and blood-pressure.

Sodium Chloride

The toxicity of sodium chloride is of special interest, due to the fact that this salt constitutes the greatest amount of salt

*Received for publication, October 14, 1936.

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present in the plasma and is largely responsible for the osmotic pressure exerted by this fluid, and also because sodium chloride plays such a vital rôle in the metabolism of the tissues.

Table I records the results obtained on respiration and blood pressure as a result of the intravenous injection of a 30 per cent solution of sodium chloride into a male dog weighing 18.18 kilos. It will be noted that the initial injection of 300 mg of sodium chloride produced an elevation of the blood-pressure from 160 to 170 mm of mercury. The respirations remained unchanged. A second injection of like amount again caused an increase in blood-pressure from 166 to 170 mm of Hg. After one minute,

TABLE I—*Effect of sodium chloride (30 per cent solution) on respiration and blood-pressure (male dog, 18.18 kg).*

Time	Blood-pressure (mm of Hg)	Respirations (per Minute)	Amount Injected (gm)
2.50	150	20	
2:59	160	22	0.300
2:59:30	170	22	
3:00	164	23	
3:01	166	23	0.300
3:02	170	22	1.500
3:02:15	178	22	
3:02:30	150	22	
3:02:45	180	25	
3:03	176	25	3.000
3:03:30	120	50	
3:04	186	26	
3:05	184	25	3.000
3:05:15	160	51	
3:05:30	180	51	
3:05:45	120	50	
3:06	184	50	
3:07	184	50	3.000
3:07:30	160	56	
3:09	180	50	4.500
3:09:30	160	56	
3:10	180	56	6.000
3:12	170	56	9.000
3:13	140	56	
3:14	172	50	15.000
3:15	124	42	
3:16	146	56	15.000
3:18	96	50	
3:19	100	Ceased	
3:19:30*	60	Ceased	
3:22†	30	Ceased	
		Total	60.600

*Heart-beat strong but slow.
†Heart-beat ceased.

the injection of 1.5 gm of the salt caused a further increase of blood-pressure to 178 mm of Hg. A two-minute interval was allowed to elapse, during which the blood-pressure fell to 150 but rose immediately to 180 mm of Hg, dropping back in a short time to 176. During this time the respirations were not significantly affected, although there is noted a gradual increase from 20 per minute to 25 per minute. Subsequent injections, however, produced marked effects upon the respiratory rate. The next injection of 3 gm of salt caused a 100 per cent increase in the number of respirations per minute, although the rate quickly dropped back to the previous rate of 25 per minute. The immediate effect of this injection upon blood-pressure was to cause it to fall to 120 mm of Hg, but it quickly returned to the level at the time of injection, namely, 184 mm of Hg. Subsequent injections had a marked effect upon respiration, causing it to be maintained at a high rate until the lethal effect was produced and caused some fluctuation in the blood-pressure, which in general was maintained at a relatively high point as compared with the preliminary level. The lethal dose of sodium chloride for this animal was 60.6 gm. Autopsy revealed edema and hemorrhage of the lungs and liver.

The results observed in this experiment can perhaps be explained by assuming that there are at least three ways in which the salt exerts its effect:

1. The osmotic pressure effect, resulting from the introduction of the hypertonic solution into the blood at first caused a withdrawal of fluid from the tissues into the blood-stream, which thereby increased the blood-volume and thus the blood-pressure. Later, the direction of fluid passage was from the vessels to the tissues.

2. There was a direct effect upon the medullary centers, which was probably an ionic effect. The general effect was to stimulate the vasomotor and respiratory centers until the lethal dosage was reached, when a collapse or paralysis of those centers occurred.

3. The third probable effect was one of cell permeability. The capillary cells became more permeable and allowed fluid to escape into the tissue spaces, which tended to cause a concentration of the blood and thereby a decrease in volume with a fall in blood-pressure. However, the stimulating effect on the vasomotor center was able to maintain the blood-pressure at relatively high levels in spite of the passage of fluid into the tissue spaces. This increased permeability of the capillaries was indicated by the edema and hemorrhage in the lungs and liver.

SODIUM CITRATE

The toxicity of sodium citrate is of interest, because sodium citrate is frequently used in blood transfusions as an anticoagulant and is perhaps the most popular anticoagulant for this purpose in use today. Petersen, Hewitt, Boyd and Brown[1] were able to produce a condition of hypocalcemia in cows, which closely simulated parturient paresis, by the intravenous administration of 20 per cent solution of sodium citrate. This condition could

TABLE II—*Effect of sodium citrate (20 per cent solution) on respiration and blood-pressure (male dog, 5.49 kg).*

TIME	BLOOD-PRESSURE (MM OF HG)	RESPIRATIONS (PER MIN.)	AMOUNT INJECTED (GM)	PULSE PRESSURE (MM)	REMARKS
3:39	132	28		2	
3:47	135	28	0.20	2	
3:47:15	103			2	R. Temporarily ceased
3:48	126	35		2	
3:49	132	28	0.10	2	
3:50:15	122	40		2	
3:50:30	128	30		2	
3:51	130	30	0.20	2	
3:51:15	112	50		4	
3:51:30	123	28		2	
3:53	123	63			R. Decreased in amplitude
3:54	130	49	0.30	2	
3:54:15	100			2	R. Temporarily ceased
3:55	128	42		2	Gasping and dyspnea, tetany
3:57	124	70		2	Tetany ceased
3:58	126	56	0.20	2	
3:58:15	103			2	R. Temporarily suspended. Tetany
3:59	122	70		2	R. Shallow, dyspnea
4:00	122	66	0.20	2	R. Increased in amplitude. Dyspnea decreased
4:00:15	82			2	R. Temporarily ceased
4:01	120	50		2	R. Shallow, irregular. Dyspnea intervals suspended
4:03	122	40	0.40	2	R. Regular and increased amplitude
4:03:30	52				Heart weak. R. suspended
4:04	110	28			R. Irregular. Dyspnea
4:05	120	25	0.40		R. Irregular. Dyspnea
4:05:15	40				R. Suspended. Heart feeble. Death
		Total	2.00		

R. = respirations.

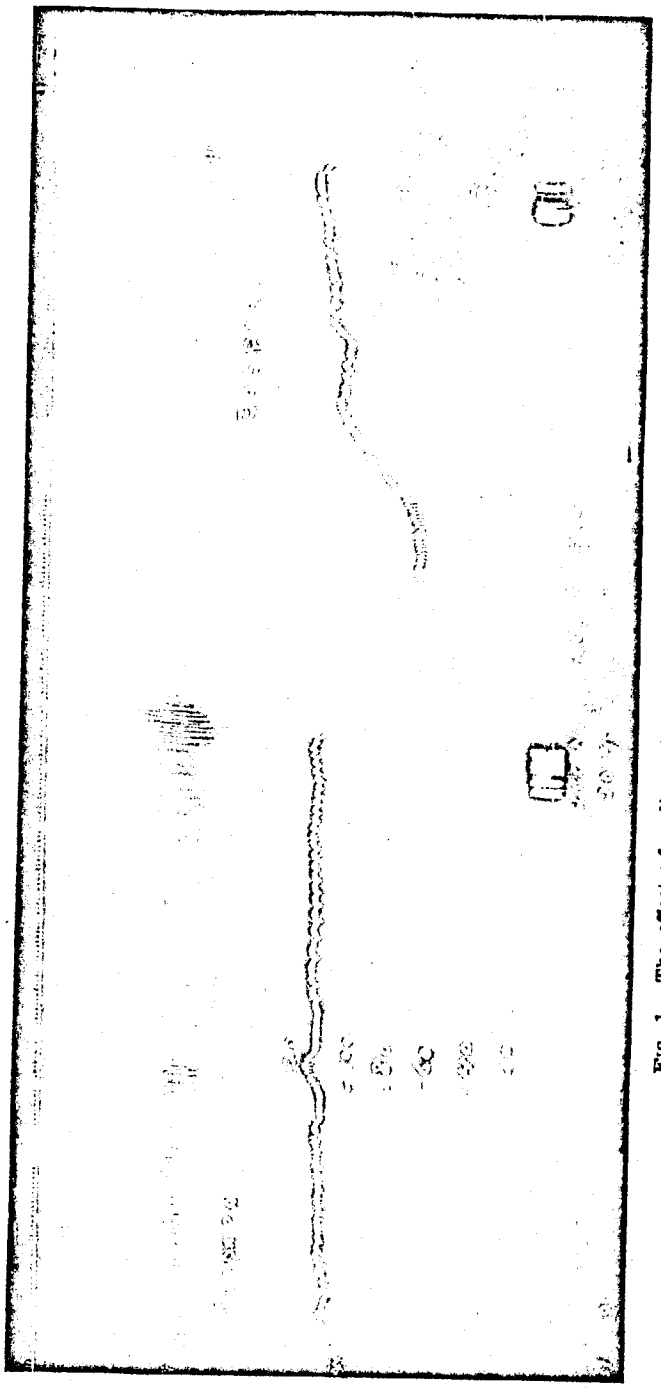

FIG. 1. The effect of sodium citrate on respiration and blood-pressure.

be immediately corrected by the intravenous administration of a 20 per cent solution of calcium chloride. They also produced tetany and prostration in rabbits by injecting 2 cc of an 8 per cent solution of sodium citrate into the auricular vein. The intravenous injection of an equivalent amount of an 8 per cent calcium chloride solution restored the animals to normal within one minute. The authors concluded that the sodium citrate formed a nonionizable calcium citrate which interfered with the mobilization of calcium and produced the condition of tetany, prostration and severe expiratory dyspnea, dilatation of the pupils and, finally, paralysis.

Table II records the results obtained on respiration and blood-pressure as the result of the intravenous injection of a 20 per cent solution of sodium citrate into a male dog weighing 5.49 kg. The initial injection of 0.20 gm caused a fall in blood-pressure from 135 to 103 mm of Hg and a temporary paralysis of the respiratory center. These effects were transient. The second injection of 0.10 gm caused a decrease in blood-pressure from 132 to 122 mm of Hg but an increase in the respiratory rate from 28 to 40 per minute. The third injection of 0.20 gm produced a similar effect; namely, it reduced the blood-pressure from 130 to 112 mm of Hg and increased the respiratory rate from 30 to 50 per minute. The pulse pressure or the difference between the systolic and diastolic pressure was increased from 2 to 4 mm of Hg. Subsequent injections produced in each case a fall in blood-pressure and a temporary holding of the respirations. Tetany and dyspnea appeared after the fourth injection, or after 0.8 gm had been injected. The lethal dose of sodium citrate was 2.0 gm. In general it can be said that this salt has a depressing effect upon blood-pressure and respiration, probably exerting its effect upon the medullary centers. To what extent this is a direct effect of the sodium citrate upon the centers or an indirect effect resulting from the displacement of calcium, it is impossible to state.

Potassium Chloride

Because the presence of potassium ions is essential to the vital functioning of various tissues, the toxicity of potassium chloride is of special interest.

Table III shows the toxic effect of potassium chloride on respiration and blood-pressure as the result of the intravenous injection of a 5 per cent solution into a female dog, weighing 10 kg. Small doses (0.075 gm) caused a rise in blood-pressure. Following the third injection, or a total of 0.250 gm, the blood-

pressure rose to 218 mm of Hg. The lethal dose was 0.850 gm. Following the last injection, which consisted of 0.200 gm, the blood-pressure rose from 180 to 192 mm of Hg and an increase in the respiratory rate from 17 to 49. This was followed in 15 seconds by a rapid fall in blood-pressure to a minimal level of 40 mm of Hg and cessation of respirations with the death of the animal. A postmortem examination of the animal showed the heart to be in a relaxed or dilated state, indicating that it had stopped in diastole (potassium inhibition).

The toxicity of potassium citrate is of interest, because of the similarity it bears to sodium citrate. Potassium citrate is also an anti-coagulant but is not recommended for transfusion purposes.

Table IV gives the results of a 20 per cent solution of potassium citrate injected intravenously into a female dog weighing 8 kg. It is to be noted that in the preliminary record, before the administration of the salt, there was a fall in blood-pressure from 180 to 150 mm of Hg. This fall was due to a venous hemorrhage, which resulted from a perforation of the femoral vein. No significant change in respiration or blood-pressure was apparent as a result of the injection of 0.04 gm of potassium citrate. However, a second injection, constituting 0.10 gm,

TABLE III—*Effect of potassium chloride (5 per cent solution) on respiration and blood-pressure (female dog, 10 kg).*

Time	Blood-pressure (mm of Hg)	Respirations (per Minute)	Amount Injected (gm)
8:47	164	14	
8:50	164	14	0.075
8:50:30	182	36	
8:52	170	14	0.075
8:52:30	180	30	
8:53	174	12	0.100
8:53:30	218	48	
8:55	178	12	0.100
8:55:30	188	45	
8:57	180	14	0.150
3:57:15	186	49	
8:58	178	15	0.150
8:58:15	184	56	
9:00	180	17	0.200
9:00:15	192	49	
9:00:30	40	18*	
9:01:15†	40	Ceased	
		Total	0.850

*Respirations very irregular.
†Death.

caused a rise in blood-pressure from 154 to 180 mm of Hg and an increase in the respiratory rate from 170 to 210 per minute. Three minutes following this injection, the blood-pressure had fallen to 146 mm of Hg and the respiratory rate was 200 per minute. This, and subsequent injections, caused an inhibition of the respiratory center and a stimulating effect upon blood-pressure.

A fourth injection, consisting of 0.20 gm of the salt, caused the blood-pressure to rise from 140 to 208 mm of Hg and a slowing of the respiratory rate from 130 to 100 per minute. As a result of the fifth injection, which consisted of 0.40 gm (total of 0.94 gm), the blood-pressure increased from 140 to 180 mm of Hg and the respirations ceased. The animal showed marked tetany, and after cessation of respiration the blood-pressure rose to 226 mm of Hg. The respirations were spontaneously resumed after 30 seconds and gradually increased to the rate of 140 per minute. The final injection of 0.40 gm (total of 1.34 gm) caused a transient rise in blood-pressure from 130 to 142 mm of Hg and a cessation of respiration. The blood-pressure rapidly dropped to 58 mm of Hg and death ensued.

TABLE IV—*Effect of potassium citrate (20 per cent solution) on respiration and blood-pressure (female dog, 8 kg).*

Time	Blood-pressure (mm of Hg)	Respirations (per Minute)	Amount Injected (gm)
3:10	180	170	
3:26*	150	170	
3:27	150	170	0.040
3:29:30	154	170	0.100
3:29:45	180	210	
3:30	145	150	
3:32:30	146	200	0.200
3:32:45	225	100	
3:35:30	140	130	0.200
3:36	208	100	
3:37:30	140	130	0.400
3:37:45†	180	Ceased	
3:38	226	50	
3:39	123	140	
3:41	140	140	
3:42:15	130	120	0.400
3:42:30‡	142	Ceased	
3:45§	58		
		Total	1.34

*Hemorrhage from femoral vein.
†Tetany.
‡Tetany.
§Artificial respiration instituted. Animal died, respiration stopping first.

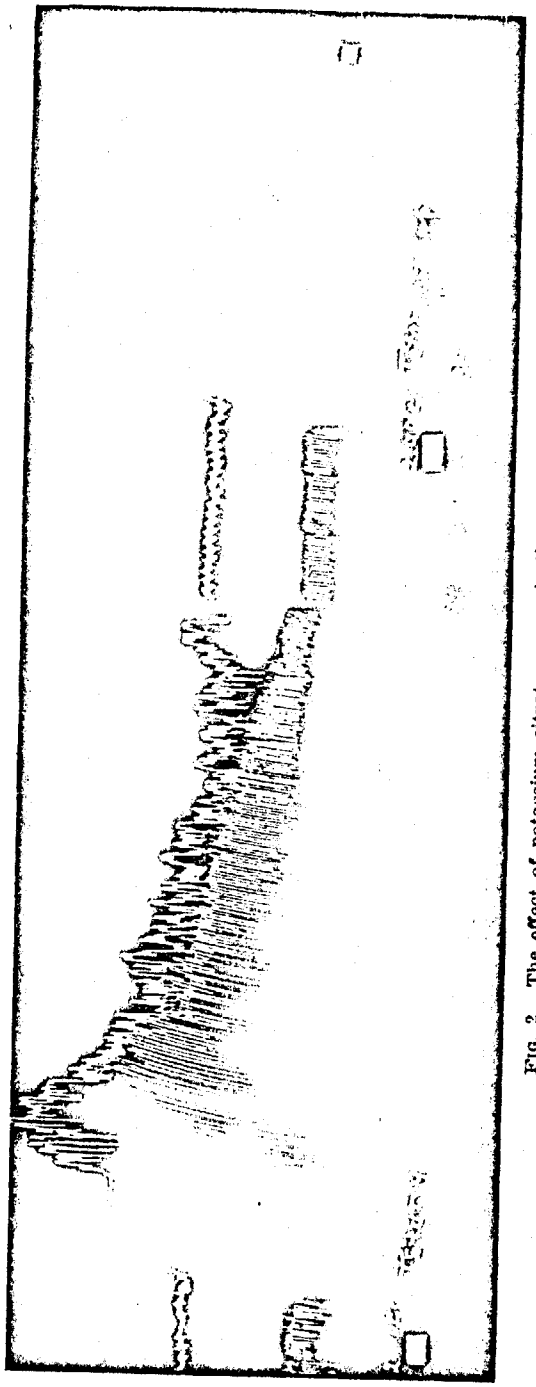

FIG. 2. The effect of potassium citrate on respiration and blood-pressure.

Calcium therapy by intravenous and hypodermic methods of administration has become an important clinical procedure in the treatment of a number of diseases. Calcium chloride is very readily soluble and ionized, which causes its effects to be produced very quickly. On the other hand, calcium chloride is quickly eliminated from the blood. Calcium chloride is one of the most toxic of the calcium salts and will cause sloughing even if only a few drops enter the tissue, hence the necessity of intravenous administration.

Table V records the effects of a 20 per cent solution of calcium chloride injected intravenously into a female dog weighing 9.09 kg. A preliminary injection of 0.20 gm of calcium chloride produced a rise of blood-pressure from 100 to 136 mm of Hg and a slowing of respirations from 32 to 26 per minute. A second injection of 0.20 gm produced a similar effect, namely, a rise in blood-pressure from 118 to 142 mm of Hg and a slowing of respirations from 28 to 21 per minute. Similar effects were noted with each injection. The heart-beats became irregular and were peculiar in having an irregular rhythm, characterized by a very strong beat occurring every five to ten seconds. This strong beat produced a pulse pressure of 50 mm of Hg, which was the difference between the systolic and diastolic pressures. The

TABLE V—*Effect of calcium chloride (20 per cent solution) on respiration and blood-pressure (female dog, 9.09 kg).*

Time	Blood-pressure (mm of Hg)	Respirations (per Minute)	Amount Injected (gm)	Remarks
2:31	105	35		
2:36	100	32	0.200	Peculiar heart-beat. Regular pulse pressure
2:36:30	136	26		
2:37	123	26		
2:38	118	21	0.200	Irregular heart-beat disappeared
2:38:30	142	28		
2:38:45	125	21		Periodic, irregular beat reappeared but less frequently
2:41	120	28	0.200	
2:41:30	145	21		
2:42	122	21		
2:43	130	21	0.400	
2:43:30	140	28		Pulse pressure 60 mm.
2:44	18	50		Heart stopped. Respirations continued for about 1 minute after
2:55	18	50		Heart stopped
		Total	1.000	

TABLE VI—*Effect of calcium gluconate (20 per cent in 3 per cent boric acid) on respiration and blood-pressure (male dog, 20 kg).*

Time	Blood-pressure (mm of Hg)	Respirations (per Minute)	Amount Injected (gm)	Pulse Pressure (mm)	Remarks
3:00	152	16			
3:04	152	21	0.10		
3:04:30	140	35			
3:04:45	156	35			Decrease in amplitude
3:06	146	42	0.10		
3:06:30	150	56			
3:08	150	50	0.20	16	Increased strength of heart-beat
3:11	162	42			
3:13	157	40	0.20		BP. and R. remain fairly constant for next 2 minutes
3:15	160	50	0.40		Rhythmic periodic increase in pulse rate
3:17	162	56	0.40	18	
3:19	170	56	0.60	20	R. weak in amplitude
3:22	172	50		40	
3:23	174	50	0.60	40–50	
3:26	170	70	0.80	10	
3:26:30	185	70		28–30	
3:30	180	63	1.00	18	R. shallow
3:30:30	197	63		30–35	
3:32	180	56	1.00	20	R. shallow and weaker
3:34	184	36	2.00	18–20	
3:35	210	28–54			
3:36	190	70	3.00		
3:37	224	63		20–46	
3:37:30	216	21		64	R. very marked increase in amplitude
3:38	210		4.00	70	
3:40	217	49			
3:43	225	35	4.00	18	
3:45	210	25	5.00	16	
3:47	220	50	6.00	16–18	
3:50	220	28	10.00	18	
3:52	182	30			R. marked increase in amplitude
3:54	203	28	10.00	16	
3:55	183	30			R. marked increase in amplitude
4:00	210	26	4.00	14–16	
4:25					Following the injection of 53.4 gm the animal was doing well
		Total	53.4		

BP. = blood pressure.
R. = respirations.

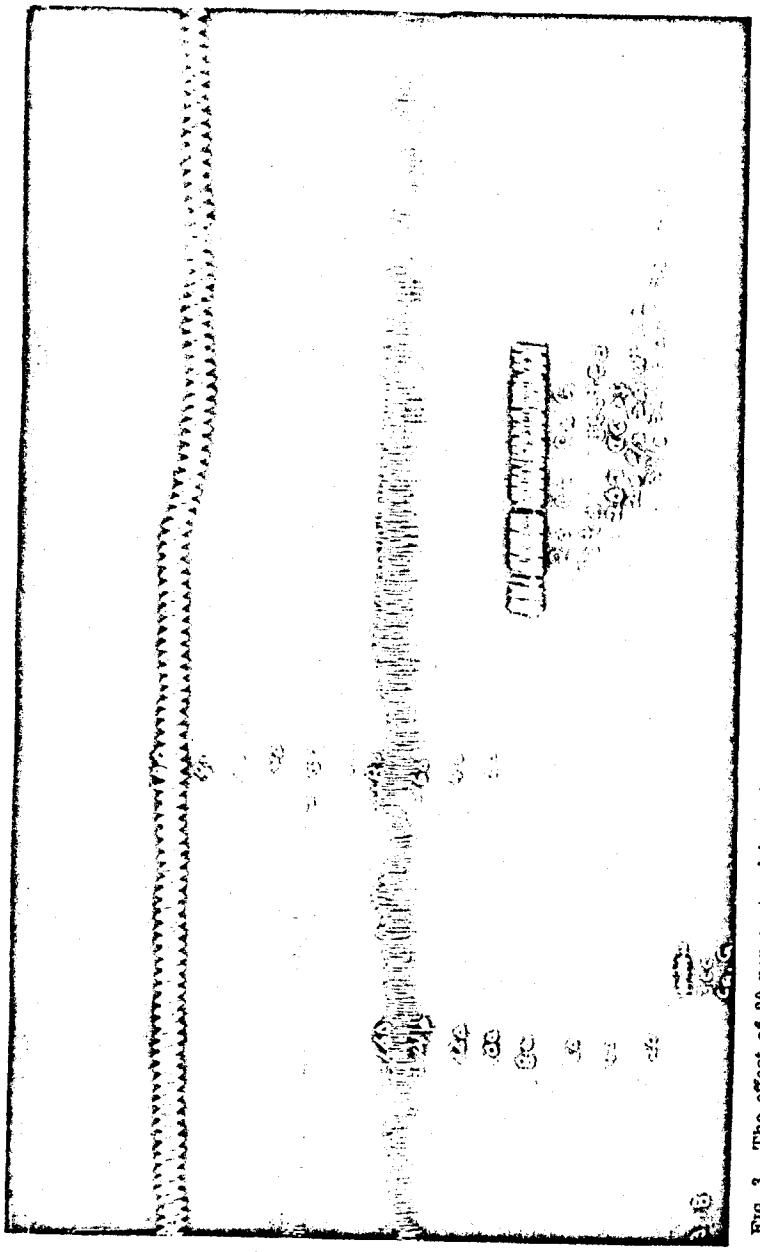

FIG. 3. The effect of 20 per cent calcium gluconate. This figure shows the effect of 0.20 gm of calcium gluconate on respiration and blood-pressure measured from the bottom base-line. It also shows the effect of a total of 53.4 gm of calcium gluconate on respiration and blood-pressure measured from the second base-line, which is the second line from the bottom.

lethal dose was 1.0 gm injected over a period of seven minutes. The last injection (0.4 gm) caused a temporary rise in blood-pressure to 140 mm of Hg followed by a sudden drop to 18 mm. The rate of respirations increased from 21 to 28 and later to 50 per minute. This increase is probably due to a reflex stimulation of the respiratory center, due to the failure of the circulatory system, producing asphyxia of the tissues. Autopsy of the animal revealed that the heart had stopped in a contracted state (calcium rigor).

Table VI records the data obtained on respiration and blood-pressure as the result of the intravenous injection of a 20 per cent solution of calcium gluconate dissolved in 3 per cent boric acid solution into a male dog weighing 20 kg. The total injection of 53.4 gm over a period of one hour in doses varying from 0.10 to 10.0 gm failed to cause the death of the animal. The blood-pressure was 152 mm of Hg at the beginning of the experiment and 210 at the end of one hour and 25 minutes.

Table VII records the results obtained on respiration and blood-pressure as the result of the intravenous injection of a 30 per cent solution of calcium gluconate into a male dog weighing 13.63 kg. In this case, the total injection of 15 gm, in doses varying between 1.5 and 4.5 gm over a period of 18 minutes, caused the heart and respirations to cease, the heart stopping one minute before the respirations.

TABLE VII—*Effect of calcium gluconate (30 per cent solution in 4 per cent boric acid solution) on respiration and blood-pressure (male dog, 13.63 kg).*

Time	Blood Pressure (mm of Hg)	Respirations (per Minute)	Amount Injected (gm)	Pulse Pressure (mm)	Remarks
2:48	156	21		6	
2:53	145	28	1.50	8	
2:54	154	35	1.50	10	
2:56	154	40	3.00	8	
2:57	150	45	4.50	8	
2:58	100	63		4–6	R. shallow
3:00	162	54		2	R. increased in amplitude
3:02	172	28	1.50	2	
3:05	180	38	3.00	2	
3:06	42	56			Heart stops. R. markedly increased in amplitude
3:07					R. cease
		Total	15.00		

R. = respirations.

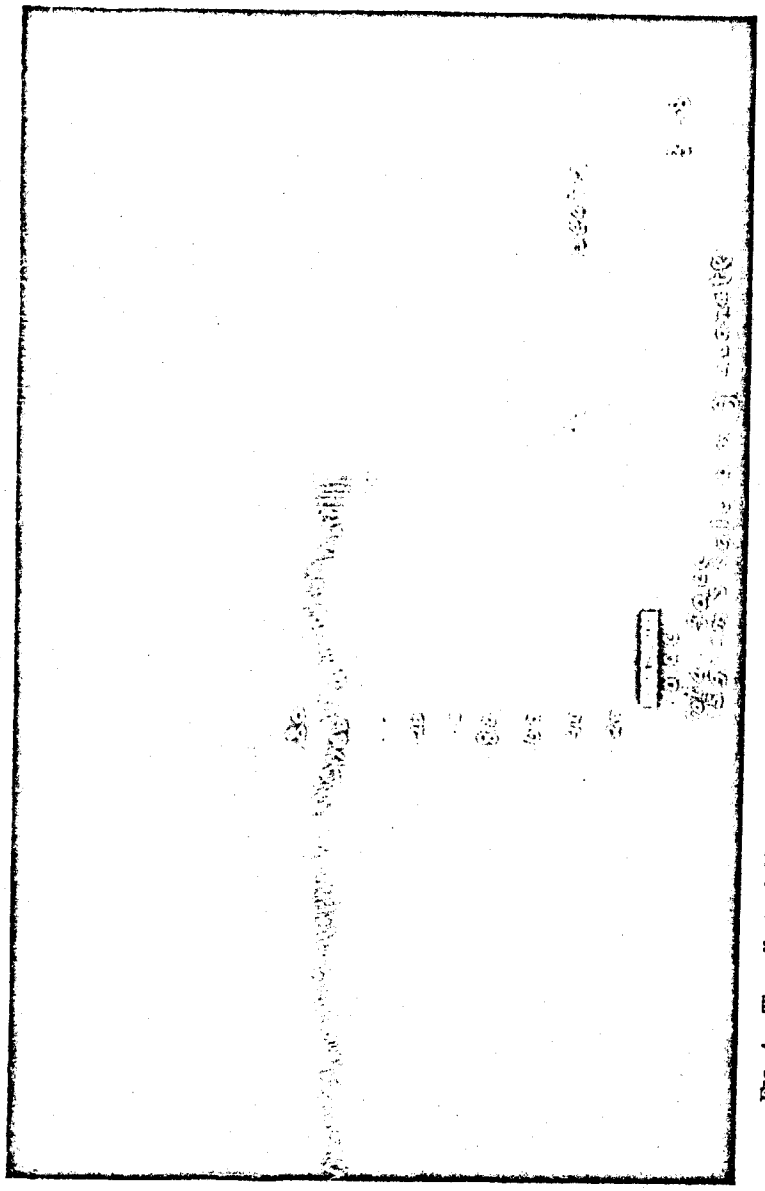

FIG. 4. The effect of 30 per cent calcium gluconate solution on respiration and blood-pressure.

Figures 1 to 4 show typical sections of tracings recording the effects of sodium citrate, potassium citrate, and calcium gluconate on respiration and blood-pressure.

SUMMARY

1. Sixty and six-tenths gm of sodium chloride in 30 per cent solution injected intravenously over a period of 17 minutes proved fatal to a dog weighing 18.18 kg. This is equivalent to 3.30 gm per kg body weight. Autopsy revealed marked edema of the lungs and liver. The observations from this experiment indicate that the toxicity of sodium chloride is effected in three ways: (1) The osmotic pressure effect, which increased blood-volume and, hence, blood-pressure; (2) a direct effect upon the medullary centers at first stimulating and later paralyzing these centers, and (3) increasing the permeability of the cell membranes, causing fluid to escape more readily into the tissue spaces.

2. Sodium citrate has a depressing effect upon blood-pressure and respiration, exerting its effect upon the medullary centers either by direct effect of the sodium citrate or by affecting the calcium. Two grams in 20 per cent solution injected over a period of 18 minutes caused the death of a dog weighing 5.49 kg. This is equivalent to 0.364 gm per kg of body weight.

3. Eighty-five hundredths gm of potassium chloride in 5 per cent solution proved sufficient to cause the death of a dog weighing 10 kg (0.085 gm per kg.) In general each administration of KCl was followed by a rise in blood-pressure and a slowing of respiration, probably through a direct action upon the medullary centers.

4. Potassium citrate in toxic doses has an inhibitory effect upon respiration and a stimulating effect upon blood-pressure. One and thirty-four hundredths gm was the lethal dose for a dog weighing 8 kg or 0.167 gm per kg of body weight.

5. One gram of calcium chloride proved to be a lethal dose for a dog weighing 9.09 kg or 0.11 gm per kg. The injections produced an irregular heart-beat with the heart stopping in systole.

6. Fifty-three and four-tenths gm of calcium gluconate in 20 per cent solution injected over a period of one hour failed to produce toxic symptoms in a dog weighing 20 kg; whereas, when a 30 per cent concentration was employed, 15 gm proved to be a lethal dose for a dog weighing 13.63 kg. In other words, when a 20 per cent solution was used, 2.67 gm per kg failed to produce

toxic symptoms; but when a 30 per cent solution was used, 1.10 gm per kg proved fatal.

REFERENCE

[1]Petersen, W. E., Hewitt, E. A., Boyd, W. L., and Brown, W. R.: Artificially induced hypoglycemia and hypocalcemia in the cow and the relationship to parturient paresis or milk fever. Jour., A. V. M. A., lxxix (1931), n. s. 32 (2), pp. 88-93.

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X-RAY PROTECTION*

By G. E. WANTZ, *General Electric X-Ray Corporation, Chicago, Ill.,*
and E. J. FRICK, *Kansas State College, Manhattan, Kan.*

The veterinary profession of recent years has become x-ray conscious and rightfully so, as there is an important, necessary, useful field for its use in veterinary medicine. However, the use of the x-ray is not entirely without its hazards.

While attempting to demonstrate at an x-ray clinic at the Oklahoma City meeting of the American Veterinary Medical Association in 1935, the authors were impressed with the fact that at least two visiting veterinarians were definitely showing finger signs of early x-ray burns and were entirely ignorant of what was causing the trouble.

The purpose of this article is not to cause undue apprehension because, if x-ray apparatus is used properly and the well-known means of protection (which are simple and easily employed) are utilized, there is *no reason or excuse* for either operator or patient receiving either a temporary or permanent injury or burn. The advantages of the use of the x-ray are all too obvious. That some disadvantages also need to be considered seems apparent.

An outline of the important hazards to be understood in the application of x-ray to veterinary practice may serve to prevent some of the detrimental experience which our colleagues in the medical field have had.

There are two "hazards" commonly associated with the operation of x-ray equipment, one being electrical shock, and the other the damage done as the result of the cumulative effects of x-ray on the body tissues. The first danger may occur due to the necessity for high voltage at the x-ray tube for the production of x-radiation. This hazard is a real one, but is found only in those equipments of the so-called "open wire" type, in which the x-ray tube is energized from conductors not designed to give high voltage protection.

This fact should be considered when one is tempted to purchase the many second-hand, non-shock-proof, non-portable equipment, on the market. Such apparatus is not safe from an electrical standpoint, either to the operator or the patient, and is not satisfactory for veterinary practice.

*Received for publication, March 6, 1937.

A Study on Sugar Metabolism, Tissue Respiration and Activity of Cholinesterase during Cardiac Arrest induced by Cardioplegic Agents.

By Richu Horigome, First Department of Surgery, Nagoya University of Medicine (Director: Professor Yoshio Hashimoto).

(The manuscript was received on October 7, 1959).

(Refer to the original text on pp. 1079-1080, for a brief English symopsis.

The essence of this thesis was made public before the Assembly and the Deliberative meetings of the following academic societies; In deptember 1958, at the 11th annual meeting of the Chest Surgery Society of Japan. In November 1958, at the second Deliberative meeting of the Committee on Cardiac Surgery under Direct Observation. In March 1950, at the 59th annual meeting of the Surgical Society of Japan. In June 1969, at the second meeting in the Kansai District of the Chest Surgical Society of Japan.

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Chapter 1: Introduction

The cardiac respiration which continues even during open hearts surgery when a heart operation is carried out under direct observation is stopped by the electrive cardiac arresting method by using various medicines in the past because it creates such problems as difficulty in surgical operation, the blocking of air cocks, the loss of a large amount of blood and the hypoxia of the myocardium, etc.

In other words, Bjork (8) attempted at the cardiac arrest in 1948 by using acetylcholine (which will hereafter be abbreviated as Ach) and Swan (2) attempted at the cardiac arrest by using potassium in 1954. Finally in 1956, Kolf and Effler (12) succeeded in its clinical application by using potassium citrate (which will hereafter be abbreviated as K. cit) and Lam (18) succeeded in its clinical application by using Ach. in1956.

On the other hand, in view of the fact that an observation of the electrocardiograms at the time of an adjusted low blood pressure by using adenosine triphosphoric acid (which will hereafter be abbreviated as ATP) revealed the existence of an elongation of the R-R distance, we attempted at its use in the elective cardiac arrest. Since 1957, we have carried out an experimental examination ofits sodium salts (which will hereafter be abbreviated as ATP-Na) and potassium salts (which will hereafter be abbreviated as ATP-K) (21).

Thus, an experiment was carried out on elective cardiac arrests by using ATP-Na, ATP-K, Ach and K. cit under extracorporeal circulation by using an artificial heart-lung apparatus and these three were compared and contrasted on the basis of the changes brought about in sugar (carbohydrate) metabolism tissue respiration and the activity of cholrinesterase (which will hereafter be abbreviated as ChE) during the cardiac arrest. Only in the case of ATP-Na and ATP-K which were the main object of our investigation, the change in the amount of ATP in the myocardium was examined.

Chapter 2: Reference Examination

Along with the development of biochemistry in recent years, there has been a shift from substrance metabolism to energy metabolism. Along with this development, a large number of reports have been published regarding the metabolism of the myocardium.

In a study of the metabolism of the myocardium, the aortic catheter method and the methods using the heart-lung specimens, tissue pieces and homogenates, etc. can be mentioned. Among these, the methods using tissue pieces and homogenates are convenient in detecting the changes in metabolic substances at each stage but it has the defects in that its conditions deviate too markedly from the biological area to understand the metabolic system as a whole. This defect cannot be eliminated even if the heart-lung specimens are employed.

According to the aortic catheter method, on the other hand, the concentration of a substance which enters into or which gets out of the heart can be obtained as the sum of the metabolism of the myocardium. However,the change in the cells or, further,the characteristics of each of the enzyme systems

cannot be clarified. Nevertheless, the latter has the advantage of obtaining a knowledge of the stage of the metabolism of the heart under more biological conditions as compared with the former method. For this reason, it seems that the aortic catheter method has mainly been used in the study of the metabolism of the myocardium in the past.

When a cardiac arrest is achieved, however, aortic flow is shut off. Thus, in the study of metabolism by means of the catheter method, an aortic "irrigation" flow is carried out only at a certain point during a cardiac arrest and, moreover, most of the contents are electrolytes, with those dealing with the carbohydrate metabolism being small in number.

Bentall (4) carried out an aortic "irrigation" flow of the cardiac arrest for a short prescribed period of time in his cardiac arresting experiment and made the following statements on the basis of the change in the amount of lactic acid in the irrigation flow;

(1) The metabolism of the myocardium during a cardiac arrest is maintained in the anaerobic sugar-decomposition stage leading to the production of lactic acid,

(2) Accordingly, it is necessary for a proper amount of glycogen to be stored in the myocardium. In thisrespect, the addition of dextrose to the K. cit-added blood which is used in the cardiac arrest is meaningful as an increase in the amount of a power-source substance.

Regarding the above, Jordan et al. (15) also stressed the importance of dextrose as a dilutant of K. cit, carried out an aortic "irrigation" flow with 10 per cent sodium lactate in the re-starting of the cardiac pulsation and observed the fact that an effective heart contraction of a regular rhythm was obtained immediately. They stated that, therefore, lactic acid was effective in the intoxication by potassium.

In the case of the tissue respiration of the myocardium, the following conclusion was obtained by Bing (5) on the basis of the result of an experiment which he carried out by using the catheter method:

(1) The amount of oxygen intake by the myocardium increases gradually up to two hours after the start of a cardiac arrest. Unless there is an injury to the respiratory enzyme system, therefore, it is believed that the myocardium is in the state of oxygen debt at the time when the aortic current is stopped.

(2) In actual fact,the irreversible termination of the heart activity is not due to the degeneration of the contractive protein (actiomyn) of the myocardium or the death of the respiratory enzyme but it is due to a change in the cell membrane of the nuclear fibers accompanying the loss of the regular stimulus transmission and rhythm.

It has been widely known since Nachmansohn's report (20) that Ach is the so-called neurohormon which plays a substantial role in muscular contraction. Regarding the question as to why Ach shows a dual function (one opposing the other, as one is stimulative and the other being inhibitive) upon the heart, Burn (10) stated on the basis of his experiment on domesticated rabbits that Ach serves stimulatively when it is administered when its concentration is low below the normal concentration and serves inhibitively when it is administered in such a manner as to achieve a concentration higher than the normal.

In addition, the report of Alles and Hawes (1) to the effect that there is a relationship between the concentration of Ach and the ChE which is its decomposition enzyme in that, when the concentration of the Ach is increased, the activity also increases but that, when the concentration goes beyond a certain level, the activity comes down is believed to support the aforementioned theory of Burn. It can be stated that herein lies the path toward the clinical application of Ach.

Based on the above observations, it is believed that an examination of the sugar metabolism, tissue respiration and ChE activity is an extremely significant and interesting matter in the comparison amongst ATP, Ach and K. cit. As for the method, the myocardiac tissue or homogenates were used even though they might tend to deviate from the biological conditions.

Chapter 3: Experimental Method (relative to extracorporeal circulation).

Grownup dogs whose weights were in the range between nine and 15 kilograms

were used in the experiment. In all cases, a tube was inserted into the respiratory system after vein anethesia, with the anesthesia hving been maintained with ether thereafter. In the case of extra corporaal circulation, a disc-shaped screw oxygen-adding apparatus (the IV C type of the University of Nagoya) and a pump of the Metalfinger type (Figures 1 and 3) were employed. As for the sucking cnnula, it was inserted into the upper vein from the vertical chest vein on the right-hand and from the right auricles and the blood-forwarding cannula was inserted into the artery under the left clavicle. Suction was based on the fall in the range between 40 and 60 centimeters. After obtaining sufficient balancing through partial extra corporeal circulation, the upper and lower large veins were cut off and the large artery was double shut off by a tape and an artery element on the capillary side of the large artery vessel valve. Next, a cardiac arresting agent was dissolved as shown in Tables 1 through 4 and rapidly injected into the upper portion of the left ventricle or in the starting portion of the large artery. After the start of the injection, the heart pulsation becomes milder in a gradual manner, finally achieving a state of cardiac arrest. Thus, the opening of the right ventricle was added and, immediately after the heating, the myocardium of the right ventricle wall was cut off three times between four and eight minutes after the stoppage of the pulsation for determinations.

(Insert Figure 1 on p. 1067. Injection of an cardiac arresting agent under extracorporaal circulation. 1. Fall. 2. Oxygen adding device. 3. Pump.)

(Insert Figure 2 on p. 1067. Oxygen-adding device (IV C type of the Nagoya University).)

(Insert Figure 3 on p. 1067. A Pump of the Metalfinger Type.)

The re-opening of the regular beating can be obtained by releasing the shutoff of the large artery.

Capter 4. Experimental Result

A separate chapter will be provided for the discussion of sugar (carbohydrate) metabolism, change in the amount of ATP, tissue respiration and changes in the activity of ChE activity, etc.

Section 1: State of Cardiac Arrest

In five examples using ATP-Na, the time required for a cardiac arrest was in the range between 35 and 55 seconds. In two examples out of these, contraction waves were observed in the electrocardiogram even though the state of a cardiac arrest was achieved under naked eye. (Refer to Table 1).

In five examples using ATP-K, the time required for the achievement of a cardiac arrest was in the range between 20 seconds and one minute and 20 seconds. Except for one example in which the shut-off of the main artery was considered to be insufficient, the period involved was short as compared with the case of ATP-Na. This is believed to be due to the multiplication effect of the ATP and potassium. (Refer to Table 2.)

(Insert Table 1 on p. 1067. Case of ATP-Na. a. Weight. b. Sex. c. Injection agent and its amount. d. Time required for the state of a cardiac arrest to be achieved and the state of the arrest. e. Fibrillation of the ventricles. 5. One gram of ATP-Na was dissolved in 20 cubic entimeters of raw . . . (Translator's Note: one character is unclear) . . . 5d. In a period of 40 seconds, a complete arrest was achieved under naked-eye observation, there are contraction waves in the cardiogram. 6c. Same as above. 6d. 47 seconds. 7c. Same as above. 7d. 49 seconds. 8c. Same as above (two injections). 8d. 55 seconds after the start of the second injection; contraction waves were observed inthe cardiogram. 16c. One gram of ATP-Na was dissolved in five cubic centimeters of raw . . . (Translator's Note: one character is unclear but looks like "salt") . . . 16c. 35 seconds.)

(Insert Table 2 on p. 1068. Case of ATP-K. a. Weight, kilogram. b. sex. c. Injection agent and its amount. d. Time required until the state of a cardiac arrest was achieved and the state of the arrest. e. Fibrillation of the vetricles. 1c. 500 milligrams of ATP-K was dissolved in two cubic centimeters of raw salt and 18 cubic centimeters of blood. 1d. 30 seconds. 2c. Same as above. 3c. 22 seconds. 3c. Same as above. 3d. 20 seconds. 4c. Same as above (two injections). 4d. 45 seconds after the start of the second injection. 9c. Same as above 9d. Became extremely mile in one minute and 20 sec-

onds (the shutoff of the main artery was insufficient).)

Among the three examples using Ach, the heart pulsation was stopped in 10 seconds after the start of the injection in experiment No. 10; however, contraction was observed each time an operation such as the opening of the ventricles was performed. In Experiment No. 11, there was observed an exdremely slowing; however, there was no complete stoppage. In Experiment No. 12, the heart pulsation stopped seven seconds after the start of the injection; however, the pulsation re-started when the ventricle was opened. When an injection was given once again, no stoppage of the pulsation was achieved. (Refer to Table 3).

(Insert Table 3 on p. 1068. In the case of Ach. a. Weight kilogram. b. Injection agent and amount of the injection. d. Time required for the arrest of the heart pulsation and the state of the cardiac arrest. e. Fibrillation of the ventricles. 10c. 0.2 grams of chlorinated acetyl choline was dissolved in 20 cubic centimeters of salt. 10d. Cardiac arrest was achieved in a period of five seconds (the amount of injection was five cubic centimeters). By the opening of the ventricles, pulsation started and, when re-injection was made, there was not arrest of pulsation. 11c. Same as above. 11d. Heart pulsation became slow but it did not stop completely. 12c. 0.4 grams of chlorinated acetyl choline, was dissolved in 40 cubic centimeters of raw salt, injection was carried out twice. 12d, Arrest was achieved in seven seconds. Contraction was observed each time operation such as the opening of the ventricles was carried out.)

In three examples using K. cit, the time required for the cardiac arrest to be achieved was in the range between five and 15 seconds. (Refer to Table 4.).

(Insert Table 4 on p. 1068. In the case of K. cit. a. Weight kilogram. b. Injection agent and the amount of the agent. d. Time required for the cardiac arrest to be achieved and the state of the arrest. e. Fibrillation of the ventricles 13c. Two cubic centimeters of 25 per cent K. cit was added to 10 cubic centimeters of . . . (Translator's Note: several characters are unclear)

. . . solution and the required amount was seven cubic centimeters. 13d. 15 seconds. 14c. Same as above, the required amount was 10 cubic centimeters. 14d 45 seconds. 15c. Same as above,the required amount was 12.5 cubic centimeters. 15d, 15 seconds.)

Regarding the method for the cardiac arrest, ventricular fibrillation and tissue change in the myocardium due to chemicals, etc. a detailed examination is being carried out by one of our co-researchers, Mr. Shimomura. Therefore, details will not be described in this report.

Section 2: Electro-cardiogram.

In any of the three cases involving ATP, Ach and K-cit, the contraction waves were found to have disappeared on the electro-cardiogram in agreement with the result of a naked-eye observation. Only in the case of ATP-Na, there was a case where contraction waves were observed despite the fact that naked-eye observation detected the state of cardiac arrest. The irregular contraction waves which did not accompany P waves as observed after the arrest of the pulsation is believed to be due to the surgical operation (Refer to Figures 4, 5, 6 and 7.)

(Insert Figure 4 on p. 1068. Electrocardiogram. (An example where no myocardium was cut off.) ATP-Na (II inducement). (Translator's Note: all other Japanese writings are too small to read.))

(Insert Figure 5 on p. 1068. Electrocardiogram (An example where the myocardium was not cut off). Ach (II inducement). (Translator's Note: Small writings are too small to read.) Note: Heart pulsation appeared by the opening of the myocardium and a re-injection did not produce the arresting of the heart pulsation.)

Chapter 5: Sugar (Carbohydrate) Metabolism.

Section 1: An Outline of the Sugar Metabolism

In view of the fact that there are a large number of Purkinje fibers and mitcondria in the myocardium or in terms of the morphological structure, the myocardium is different from the other medulla muscles. However, in terms of the metabolic mechanism, it is engaged in approximately the same metabolic

mechanism, it is engaged in approximately the same metabolic course. In other words, it takes the carbohydrates, fatty acids and amine acids contained in the blood, it oxidized and decomposes the intermediate metabolic products of the tissue itself, and produces, stores and synthesizes the energy in conjunection with the phosphorization reaction and, while maintaining a suitable equilibrium, it carries out its functions. An extremely large number of enzymes participate in the stage of these decompositions and syntheses.

(Insert Figure 6 on p. 1069. Electrocardiogram (an example where the myocardium was not cut off). Ach (III inducement). (Translator's Note: Other writings are too small to read).)

(Insert Figure 7 on p. 1069. Electrocardiogram (an example where the myocardium was not cut off.) K.cit (II inducement). (Translator's Note: All other writings are too small to read.))

Dextrode, lactic acid,pyrograpе acid, acetic acid, ketoacid and fatty acid, etc. can be mentioned as the sources of energy for the myocardium to work (6, 7). The main stage for the development of the said energy is the desaccbarization process. All of the stage for the glycogen to be metabolized into pyrograpе acid through dextrose-6-phosphoric acid and finally into lactic acid is carried out anaerobically and, therefore, it is called anaerobic desacchariation. The reaction which belong to this stage are considered to be reversigle.

The pyrograpе acid which is produced in this stage enters the Krebs circuit (16) under aerobic conditions and is decomposed completely into carbon dioxide and water. When it is completely oxidized to this point, 710,000 calories per molecule of the dextrose is produced. In an anaerobic desaccharization stage, only approximately 1/12 of that which is obtained in complete oxidation or 57,000 calories is produced. However, this stage is characterized by the following:

(1) It is carried out without the supply of oxygen and it is carried out extremely quickly. Therefore, it is extremely convenient to comply with the rapid demand for energy in living bodies.

(2) The energy which has been produced in accumulated and utilized as ATP at an extremely high rate (approximately 65 per cent).

(Insert Figure 8 on p. 1069. Anaerobic desaccharization stage (names of enzymes are omitted). 1. Clygogen. 2. Dextrose 1 phosphoric acid. 3. Dextrose. 4. Dextrose 6 phosphoric acid. 5. (Translator's Note: one character is unclear but looks like) levulose 6 phosphoric acid. 6. (Translator's Note: one character is unclear but looks like (levulose 6 (Translator's Note: three characters are unclear). 7. Dehydroxy acetone (Translator's Note: two characters are unclear). 8. Glycerine aldehyde (Translator's Note: two characters are unclear). 9. Glycerine (Translator's Note: four characters are unclear). 10. Glycerine 2-phosphoric acid. 11. Glycerine 2-phosphoric acid. 12. (state without oxygen). 13. State with oxygen. 14. Krebs circuit. 15. pyrograpе acid (Translator's Note: Two characters are unclear). 16. Grape acid. 17. Lactic acid.)

The anaerobic desaccharization stage will be shown in Figure 8. The reaction (4) or the reverse reaction from levulose-6-phosphoric acid to levulose-1-6-diphosphoric acid is carried out under the action of a special phosphoric acid enzyme. The lactic acid which has been produced by the reaction (12) diffused as long as it is not burnt; it is transported to the liver by the blood. In the liver, it is synthesized into glycogen or dextrose through a course which is the reverse of the desaccharization stage. Lactic acid is not oxidized as it is under aerobic conditions but is re-oxidized once into pyrograpе acid before entering the oxidation stage. When it is synthesized into glycogen, it is converted once into dextrose before it is re-synthesized. Accordingly, lactic acid is not on intermediate methabolic product but should be considered to be a final product or a stable product (33).

(Insert Figure 9 on p. 1070. Krebs circuit. 1. Acetyl CoA. Oxaloacetic acid. 2. Citric acid. 3. Cis (Translator's Note: two characters are unclear) acid. 4. Iso-citric acid. 5. Oxalo succinic acid. 6. (alpha)-ketoglutaric acid. 7. Succhel CoA. 8. Succinic acid. 9. Fumaric acid. 10. Malic acid.

11. Grape acid. The enzymes which participate in the aforementioned circuit. (1) Aconidase. (2) Aconidase. (3) Isocitric acid dehydrogenation enzyme. (4) Oxalosuccinic acid decarbonization enzyme. (5) (alpha)-ketoglutaric acid oxidation enzyme. (6) Succinic acid dehydrogenation enzyme. (7) Translator's note: writing is too small to read). (8) Malic acid dehydrogenation enzyme.)

The final stage of the oxidation decomposition of sugar (the Krebs circuit) will be shown in Figure 9. This is a stage which is common to fats and protein.

First, grape acid is converted into active acetic acid in the form of acetyl CoA by the following reaction:

$CH_3.CO.COOH$ plus DPN plus CoA go into acetyl CoA plus CO_2 plus $DPNH_2$.

Active acetic acid is reacted with the enol type of oxaloacetic acid by CoA.-ATP, thereby producing citric acid and entering the Krebs circuit. Citric acid loses water by the action of the aconidase, thereby being converted into cis aconitic acid, which is further given an addition of water due to the actions of aconidase to be converted into iso-citric acid.

It is further dehydrogenated by the iso-citric acid dehydrogenation enzyme, thereby producing oxalosuccinic acid. Next, it is decarbonated by oxalosuccinic acid dehydrogenation carbonic acid enzyme to be converted into (alpha)-ketoglutase. It is then converted into succinic acid by the (alpha)-ketoglutaric acid oxidation enzyme system. The succinic acid is converted into fumaric acid by the succinic acid dehydrogenation enzyme. Next, by the presence of fumarase, it is converted into malic acid by the addition of water. The malic acid is converted into oxaloacetic acid due to dehydrogenation by the malic acid dehydrogenation enzyme and the oxalo-acetic acid is enolated by voluntary isomerization and again enters the circuit.

The above is an outline of the Krebs circuit. In conjunction with the oxidation of the substrate in this connection, a phosphorization reaction takes place. Along with a clarification of the high-energy phosphoric acid union of ATP, it is considered to be important as a system for the grasping of respiratory energy in the living bodies.

Therefore, as means for learning something about the sugar metabolism at the time of the cardiac arrest, the glycogen, grape acid and lactic acid in the anaerobic desaccharization stage were determined. For the purpose of learning something about tissue respiration, further, changes in the activity of succinic acid oxidation enzyme (succinic acid dehydrogenation enzyme and chitochrome oxidation enzyme) were examined (details will be described in Chapter 6).

Section 2: Materials for Determination and Method of Determination.

Immediately after the cardiac arrest, four and eight minutes after the cardiac arrest, the myocardium of the right-hand ventricle was cut off. By avoiding the fat tissue and connective tissue parts, the blood components were sufficiently wiped off with filter paper. Accurately one gram of the same was weighed for use in the determination of glycogen. In the determination of lactic acid and pyro-grape acid, one gram of the myocardium was homogenized with 10 cubic centimeters of trichlorinated acetic acid. One cubic centimeter of the same was used for the measurement of the dry weight and the values were expressed in terms of 200 milligrams of dry weight.

As for the determination, glycogen was determined by the diphenyl amine method (9), pyrograpе acid was determined by the Lu and Nelson method (10, 26), lactic acid was determined by the Barker and Summerson method (3, 27) and colorimetry was carried out by the photoelectric colorimeter of Beckmann.

Section 3: Result of Determination

Clause 1: In the case of ATP-Na.

In examples where no ventricular fibrillation was developed as shown in Figure 10, the glycogen showed no change whatsoever in four minutes or was slightly reduced but was increased after eight minutes. There was observed a tendency for decrease in pyrograpе acid except for one case. It was difficult to observe a fixed tendency in the case of lactic acid.

In the examples where ventricular fibrillation was produced, glycogen was markedly reduced and pyrograpе acid was increased in amount once but was then

decreased. The amount of lactic acid was increased.

(Insert Figure 10 on p. 1071. In the case of ATP-Na. a. (Example of no ventricular fibrillation). b.(Example of ventricular fibrillation.) 1. (Glycogen). 2. (Pyro-grape acid). 3. (Lactic acid). c. Immediately after. d. Four minutes after. e. Eight minutes after. f. Immediately after. g. Four minutes after. h. Eight minutes after.)

(Insert Figure 11 on p. 1071. In the case of ATP-K. a. (Example of no ventricular fibrillation.) b. (Example of a ventricular fibrillation). c. Immediately after. d. Four minutes after e. Eight minutes after. f. Immediately after. g. Four minutes after. h. Eight minutes after. 1. (Glycogen). 2. (Grape acid). 3. (Lactic acid).)

(Insert Figure 12 on p. 1071. In the case of Ach. K. cit. 1. (glycogen). 2. (Grape acid). 3. (Lactic acid). a. Immediately after b. Four minutes after. c. Eight minutes after d. Immediately after. e. Four minutes after. f. Eight minutes after.)

Clause 2: In the case of ATP-K.

In the case of those which did not develop ventricular fibrillation, glycogen was reduced at first and was then increased. This was the same as in the case of ATP-Na. It was difficult to discover any definite trend in the case of pyro-grape acid and lactic acid. In the case of those which developed ventricular fibrillation, however, glycogen was reduced to a marked extent and it was difficult to discover any definite trend with regards pyro-grape acid and lactic acid. It is worthy of special notice that there was a reduction in lactic acid in two cases out of three.

Clause 3: In the case of Ach.

As shown in Figure 12, all three cases did not develop the dibrillation of the ventricles. In two cases involving glycogen, there was a reduction at first and then this was followed by an increase. In one example, the initial increase was followed by a decrease. It was difficult to discover a definite trend regarding pyrogrape acid and lactic acid.

Clause 4: In the case of K. cit.

As shown in Figure 12, no fibrillation of ventricles was developed in all three cases. In the case of glycogen, there was a reduction at first and an increase later. Even though there was found no definite trend regarding pyro-grape acid and lactic acid, lactic acid showed an initial increase, which was followed by a decrease at a later stage.

Section 4: Conclusion

In the experiments on cardiac arrest by using ATP-Na, ATP-K, Ach and K. cit, only in the case of myocardium glycogen was it possible to detect any definite trend that there was a decrease four minutes later and an increase eight minutes later. It was not possible to detect any definite trend in pyro-grape acid and lactic acid as in the case of glycogen. However, Figure 13 shows the determination examples of these four in the experiment where the course of some fixed pattern seems to have been taken. The trends shown here seem to be approximately the same.

(Insert Figure 13 on p. 1072. Examples of Fixed Patterns. 1. Glycogen. 2. Lactic acid. 3. Grape acid. 4. Immediately after. 5. Four minutes later. 6. Eight minutes later. 7. Immediately thereafter. 8. Four minutes later. 9. Eight minutes later. 10. Immediately after. 11. Four minutes later. 12. Eight minutes later. 13. Immediately after. 14. Four minutes later. 15. Eight minutes later.)

Chapter 6L Change in the Amount of ATP

Section 1: Significance of ATP in Living Bodies

It is believed that the ultimate source of energy which is required for the various biological stages in the life of a living body including the synthesis of various substances required for the maintenance of life and its growth, such mechanical work as the contraction of muscles, such a permeative work as at the time of the absorption and discharge of substances, and the generation of electricity in the nervous system and the generating organs, etc. is the high-energy phosphoric acid union or in the energy which is stored in the phosphoric acid union of ATP.

Regarding one gram molecule of this, an energy of approximately 12000 calor-

ies is discharged at the time when onephosphoric acid group is hydrolyzed. Accordingly, it is necessary for A TP to be reproduced by some means whatsoever from ADP which has been produced by the disappearance of the phosphoric acid group in ATP during the course of the loss of the energy in order for the life to be maintained. Regarding the synthesis of this ATP, it has been stated that two molecules of ATP is produced during the course of anaerobic desaccharization, for example, and that 30 molecules of ATP are produced in the Krebs circuit. However, there are a number of points which are not clear in the reaction for its production.

In recent years, a certain school of thought as enunciated by Szent-Gyorgyi (25) carried out a study on the mutual action between actmyocin and ATP and arrived at the contraction cycle which is the reaction mechanism amongst actmyocin in muscular contraction, ATP and salts. However, the present stage of development is such that no concrete theory has been established regarding the mechanism as to the conversion of the chemical energy in the ATP molecules into mechanical energy in the case of muscular contraction and the true state of the contractible substance existing in the muscles. In any event, there is no question whatsoever as to the important role played by ATP in muscular contraction.

Therefore, we examined the change in the amount of the ATP in the myocardium at the time of cardiac arrest by ATP-Na and ATP-K, immediately thereafter and eight minutes thereafter.

Section 2: Materials for Determination and Method of Determination.

(Insert Figure 14 on p. 1072. Change in the amount of ATP (cardiac arrest was caused by ATP). F: Fibrillation of the ventricles. a. Milligrams in 100 grams of the myocardium. b. Immediately afterward. c. Eight minutes afterward.)

The myocardium of the walls of the right-hand ventricle which was cut off immediately after the achievement of cardiac arrest and eight minutes after the attainment of cardiac arrest was employed.

For determination purposes, column chromatography using ion exchange resin,

which was for the first time used by Cohn and Carter (11) in their analysis of adenosine polyphosphate was used. Among a large number of methods for determination, this method which has recently been developed to a marked extent is said to be highly suitable for the determination of adenosine polyphosphate in the muscles. Here, the author used the method and modified by Miyazaki and Uchida, et al. (30, 32). Details of this apparatus, the extraction method, reagents, experimental operations and the formulas of calculation, etc. are omitted from this report.

Section 3: Result of Determination

As shown in Figure 14, there was observed an increase in amount in the range between 20 and 30 per cent eight minutes later as compared with the values obtained immediately afterward both in ATP-Na and ATP-K, except for the cases where the fibrillation of the ventricles was developed. There was observed a reduction in all cases where there was the development of ventricular fibrillation.

Section 4: Conclusion

The amount of increase in ATP will be discussed again at a later stage (Section dealing with Observations) inasmuch as it is related to the stage of anaerobic desaccharization and tissue respiration. It is believed that, in the case where there was the development of ventricular fibrillation, ATP participated in the production of energy, with a result that its amount was reduced.

Chapter 7: Tissue Respiration

Section 1: Tissue Respiration and Energy Metabolism

Anaerobic desaccharization is one of the basic stages of power source in living bodies. When free oxygen is given, however, the living body acquires energy by more effective means called breathing. This is clear from the aforementioned fact that two molecules of ATP is produced in the stage of anaerobic desaccharization and 30 molecules of ATP is produced in the Krebs circuit. Among the respiration enzymes which are considered to be closely related to the metabolism of the tissues, it is believed that the titochrome oxidation enzyme is related to the reaction for oxygen absorption by the succinic acid

The temperature of the constant-temperature vessel was set at 37 degrees centigrade, the number of agitations was in the range between 100 and 120 per minute, the gas was air and 2 NKOH was charged into the central auxiliary chamber in the amount of 0.2 cubic centimeters so that the carbon dioxide which was produced by breathing would be absorbed. In addition to the pressure gauge for testing purposes, a control gauge was used as a temperature manometer for the purpose of correcting the errors in the readings of the test pressure gauge stemming from the change in the temperature and atmospheric pressure inside the gas cavity. Under the conditions of constant temperature and constant capacity (volume), a reduction stemming from the absorption of oxygen was read on the pressure gauge and the amount of oxygen (c. millimeter) which was absorbed was calculated by using the prescribed formula.

In a comparison of tissue respirations, Warburg used the following formula: QO_2 = Amount of oxygen absorbed (c. mm) divided by (the dry weight of tissue (mg) x time (hr).

Since the conditions were constant in our experiment, however, no conversion was made to QO_2 and a comparison was made by using the amounts of oxygen absorption at 40 minutes where the reaction progressed approximately lineally. (Reference should be made to Figure 15.)

(Insert Figure 15 on p. 1074. Succinic acid oxidation enzyme O_2 uptake curve (c. mm/20 mg. dry weight). a. Control.)

Section 5: Result of the Experiment

As a control, three regular (normal) dogs were used. The myocardium muscle of the right-hand ventricle which was in a pulsation state under anesthesia induced by a tube insertion was cut off and the amount of their oxygen intake was measured. Table 5 shows the change in each individual case as compared with the control in percentage.

(Insert Table 5 on p. 1074. Succinic acid oxidation enzyme O_2 uptake (c. mm/20 dry weight)(values without the autoxidation portion). a. Control. b. Experiment No. c. 10 minutes. d. 20 minutes. e. 30 minutes. f. 40 minutes. g. Rate of change, per cent. h. Average of the 40-minute values in three exam-

ples. ATP-Na 7, above: immediately afterward. ATP-Na 7; below: eight minute afterward. 8, above: immediately afterward, below: eight minutes afterward. 16, above: immediately afterward, below: eight minutes afterward. Ach, 10, above: immediately afterward, below: eight minutes afterward. 11, above: immediately afterward, below: eight minutes afterward. 12, above: immediately afterward, below: eight minutes afterward. K. cit. 13, above: immediately afterward, below: eight minutes afterward. 14, above: immediately afterward, below: eight minutes afterward. 15, above: immediately afterward, below: eight minutes afterward.)

In the case of ATP-Na, the ranges were between plus 22 per cent and plus 33 per cent immediately afterward and between plus 29 per cent and plus 41 per cent eight minutes afterward.

In the case of Ach, the range was between plus five per cent and plus 34 per cent immediately afterward and between plus 11 per cent and plus 42 per cent eight minutes afterward.

In the case of K. cit, the range between zero and plus 18 per cent immediately afterward and between plus 13 per cent and plus 24 per cent eight minutes afterward.

Section 6: Conclusion

In all cases of ATP-Na, Ach and K. cit, the activities of the myocardium succinic acid oxidation enzyme were larger than that of the control. Their order was as follows:

ATP-Na more or less similar to Ach, which was greater than K. cit, which was greater than the control.

As compared with the values obtained immediately afterward, the activities obtained eight minutes afterward were greater.

Chapter β: Change in Cholinesterase Activity

Section 1: Significance of Cholinesterase

At the time when the heart is active, an activity current is produced in accompaniment of a stimulus transmission and muscular contraction. Such a biological electric phenomenon is related to the production and disappearance of

Ach (14).

A shift of ions is produced by a change in plasma membranes based on the production of Ach and the union with its receiving protein. As a result, it is believed that biological electricity is produced (20).

(Insert Figure 6 on p. 1074. Mechanism of the Function of Acetylcholine. 1. Union type. 2. Proteins. 3. Esterase. 4. Choline acetylase. 5. Choline. 6. Acetic acid. 7. Active acetic acid. 8. (Translator's Note: unclear but looks like) acetoquinase. 9. Ionic shift. 10. Receiving protein. 11. Desaccharization. 12. Cr phosphoric acid.)

According to Nachmansohn, there is no action by Ach of the union type as shown in Figure 16. However, he states that, when it receives a stimulus, this Ach is freed. This freed Ach is united with the receiving protein which constitutes the plasma membrane, thereby changing the structure of the membrane and causing the sodium ions to move. On the other hand, the Ach is immediately decomposed into choline and acetic acid by ChE. The choline and acetic acid which have been produced are re-synthesized by choline acetylase. In this recovery stage, the creatin phosphoric acid which was produced in the stage of desaccharization supplies energy through ATP.

Therefore, the change in the activity of ChE which is the decomposition enzyme for Ach was examined by using the Warburg pressure gauge at the time of a cardiac arrest by ATP, Ach and K. cit.

Section 2: Experimental Method

In order to measure the activity of ChE, there are a number of methods including a biological method and a chemical titration method, etc. The one which is most frequently employed is the method using a gas measurement. Improvements have been made by a number of researchers on the method which was used by Ammon who used the Warburg pressure gauge (2). In this experiment, the method (13) which was described by Glick was used.

Immediately after the achievement of a cardiac arrest and eight minutes after the attainment of a cardiac arrest, the myocardium muscle was cut off,

These were made into homogenates by using five cubic centimeters of the Ringer solution. Zero point five (0.5) cubic centimeters of the same was used for pressure detection and one cubic centimeter was used for a measurement of its dry weight.

Principle: By the acid which is decomposed and freed from the ester of choline, the carbon dioxide which was produced fromthe sodium bicarbonate was measured.

The composition of the bicarbonate Ringer solution (pH 7.4): 0.9 per cent NaCl 100 cubic centimeters, 1.26 per cent $NaHCO_3$ 30 cubic centimeters, and 1.76 per cent $MgCl_2.6H_2O$ two cubic centimeters.

This solution was saturated with a mixed gas consisting of 95 per cent N_2 and five per cent CO_2 before it was put to use. One cubic centimeter of the aforementioned bicarbonate Ringer solution and 0.5 cubic centimeters of the myocardium homogenate were charged into the main chamber and 0.5 cubic centimeter of 1/10M chlorinated acetylcholine was charged into the sidearm, to make the total amount 2.0 cubic centimeters. The number of agitations was in the range between 90 and 100 per minute and the temperature of the constant-temperature vessel was set at 37.5 degrees centigrade.

(Insert Figure 17 on p. 1075, Cholinesterase CO_2 output curve. a. Control).

(Insert Table 6 on p. 1075. Cholinesterase CO_2 output. a. Control. b. Experimental No. c. 10 minutes. d. 20 minutes. e. 30 minutes. f. 40 minutes. g. 50 minutes. h. 60 minutes. i. Rate of change. j. Average of the 60-minute values in three cases. ATP-Na, 7, above: immediately afterward, below: eight minutes afterward. 8, above: immediately afterward, below: eight minutes afterward. 16, above: immediately afterward, below: eight minutes afterward. Ach, 10, above: immediately afterward, below: eight minutes afterward. 11, above; immediately afterward, below: eight minutes afterward. 12, above: immediately afterward, below, eight minutes afterward. K. cit, 13, above: Immediately afterward, below: eight minutes afterward. 14, above: immediately afterward, below: eight minutes afterward. 15, above: immediately

afterward, below: eight minutes afterward.)

Section 3: Experimental Result

As in Chapter 6, the CO_2 output of the myocardium muscle of the right-hand ventricle in three objects in the control group was examined. Since these curves would progress approximately lineally up to 60 minutes (reference should be made to Figure 17) it was decided that a comparison would be made on the basis of the CO_2 output (c. mm) at 60 minutes. The result obtained is shown in Table 6.

In three cases of ATP-Na, the value obtained was approximately minus 40 per cent and there seems to be a trend for recovery eight minutes afterward as compared with the value obtained immediately afterward.

In three cases of Ach, the value obtained immediately afterward was minus 60 per cent and approximately minus 50 per cent eight minutes afterward. The trend for recovery eight minutes afterward as compared with the value immediately a fterward was the strongest amongst the three chemicals.

In three cases involving K-cit, the value obtained immediately afterward was approximately minus 25 per cent. As compared with this, there seems to be a trend for recovery eight minutes afterward.

Section 4: Conclusion

At the time of a cardiac arrest by ATP-Na, Ach and K. cit, the activity of ChE was lower than in the control in all cases. The order was as follows:

Control was greater than K. cit, which was greater than ATP-Na, which was greater than Ach.

Chapter 9: Observations

In the experiment which we carried out, since the main artery was shut off, there was no supply of various substances and oxygen to the artery blood. Under this condition, the result of the experiment obtained will be examined below:

(1) Change in Glycogen

When a muscle is contracted by means of some stimulus or when the muscle is left in the state of no oxygen, the amount of glycogen is reduced and, instead, lactic acid is accumulated. When this muscle is left in air or is made to rest

with oxygen being supplied thereto, respiration takes place, with a result that the accumulated lactic acid is reduced and that the glycogen is increased in amount.

When, at this time, the actually observed amount of the loss of lactic acid is compared with the amount of the disappearance of lactic acid as calculated on the basis of the amount of oxygen consumed, it is observed that the latter is approximately 1/6 of the former. On this basis, Meyerhof interpreted it as meaning that the lactic acid is once again re-synthesized into glycogen by the energy freed by the respiration.

According to his interpretation, the energy which is utilized in the work inside the living cells is produced by the desaccharization action. This desaccharization action takes place irrespective of whether there is oxygen or not. When there is oxygen, however, since the lactic acid is not synthesized into glycogen for accumulation, the result is that the production of lactic acid is inhibited.

A large number of studies have been added to the phenomenon of the inhibition of enzyme in the state of oxygen being in existance or the so-called Pasteur effect and a large number of theories have been made public regarding its explanation. It appears that it is not possible to completely explain the phenomenon by using only one of these theories (29, 34).

The reduction in glycogen four minutes afterward can also be considered as an extension of the glycolysis stemming from the cardiac pulsation before the cardiac arrest was made. In order to apply the Meyerhof explanation to the amount of an increase of glycogen eight minutes afterward, the requirement of a state of oxygen being in existance is not satisfied to a sufficient extent. In other words, the amount of oxygen supplied to the myocardium after the cardiac arrest has been achieved is considered to be very small since it comes from the blood in the artery existing in the myocardium and the contact of the surface part of the myocardium with air after the opening of the ventricles.

Despite the fact that there was an increase in the amount of glycogen eight minutes afterward, the amount of lactic acid was observed to have increased in

some cases.

From the reasons mentioned above, the interpretation to the effect that the amount of glycogen was increased by the reverse process of anaerobic desaccharization cannot be accepted wholeheartedly. In addition, the synthesis from dextrose in the existing myocardium is a process which is conceivable. In any event, the increase in the amount of glycogen prior to the opening of the cardiac pulsation can be considered to be one of the extremely advantageous conditions as an increase in the amount of a power-source substance.

The reduction in the amount of glycogen in the example involving the generation of ventricular fibrillation is considered to stem from its decomposition and reduction in conformity with the high degree of demand for energy from the myocardium.

(2) Change in pyro grape acid.

It has been held that, in a living body, pyro grape acid and lactic acid have a similar tendency for change under the same conditions. Such a tendency was not observed in the experiment that we carried out. As a whole, there was a trend for pyro grape acid to decrease. When the tendency for an increase in the amount of lactic acid is taken into consideration (as will be described later), it is either that there was a shift to lactic acid in the state of insufficient oxygen or that a part of the same entered the Krebs circuit for decomposition. Since pyro grape acid is an unstable intermediate metabolic product under various complicated inner conditions, it is difficult to discover such a significance in this case as in the case of glycogen or lactic acid.

(3) Change in Lactic Acid

In the case of lactic acid, where was observed a tendency for the amount to decrease once and then to increase later. This can be understood to result from the desaccharization. In an example involving the development of a ventricular fibrillation, there were cases where there was no marked increase in the amount of lactic acid but there was rather a reduction in its amount, contrary to the original expectations. (Reference should be made to Figure

11.).

This is believed to be indicative of the theory enunciated by Jordan (16) to the effect that lactic acid is effective in order to achieve an effective contraction of the heart. On the other hand, in view of the fact that there was no increase in the amount of glycogen, it is also conceivable that it entered the Krebs circuit as a source of energy to be decomposed there. However, the causal relationship as to whether the amount of lactic acid was reduced because of the fibrillation of the ventricles or a reduction in the amount of lactic acid served as a factor for the development of the ventricular fibrillation is not clear.

(4) Change in the Amount of ATP

In the example involving a cardiac arrest by ATP-Na and ATP-K, there was a marked increase in the amount of ATP eight minutes afterward as compared with the values obtained immediately afterward, except in the cases where there was the development of ventricular fibrillation. Along with the increase in the amount of glycogen, this is significant as an increase in the amount of a power-source substance.

Since the injection agent is the same substance as ATP which is determined in this case, there is presented a problem as to whether the ATP which has been injected may be included in the measured value. Since ATP itself is an extremely unstable substance, it is inconceivable that the ATP will remain as it is until the determination operation is started If it is assumed that the injected ATP is included in the determined value, it ought to be the case that the value obtained immediately afterward is greater than the value obtained eight minutes afterward. For the reasons mentioned above, it is believed proper to consider that the measured ATP is that of the myocardium itself.

The reduction in the amount of ATP in the example involving the development of a fibrillation of the ventricles is believed to be due to its participation and consumption in the production of energy. The problem as to why the ATP (23) which has an action resisting the fibrillation of the ventricles (anti-fibrillatory action) develops the fibrillation of the ventricles should be

examined in the light of other factors such as temperature, etc.

The comparison amongst the three including ATP, Ach and K. cit with the sugar metabolism as the center as presented above reveals that no marked difference exists among them and that, especially the increase in the amounts of glycogen and ATP prior to the re-opening of heart pulsation, can be considered as indicative of the possibility of an elective cardiac arrest method from the standpoint of energy metabolism.

In the example involving the development of ventricular fibrillation, further, there were cases where the amounts of glycogen and ATP were reduced and even the amount of lactic acid was reduced. It goes without saying from the standpoint of a reduction of these power-source substances that the ventricular fibrillation should be avoided.

(5) Tissue Respiration

In all cases, the activity of the myocardium succinic acid oxidation enzyme which is considered to be in parallel relationship with the tissue respiration in the case of a cardiac arrest by ATP-Na, Ach and K. cit showed a tendency of being greater than in the case of the control. In view of the fact that the activity was greater eight minutes afterward than immediately afterward, it seems proper to be the debt of oxygen based on "crest blood current shut-off" (aortic acclusion) as stated by Bing. This is also in support of the statement of Bentall (4) to the effect that the stage of desaccharization is anaerobic during a cardiac arrest and it is also indicative of the fact that there is hardly any obstacle to the respiratory enzyme system.

Even though it is not clear to what chemical action the difference amongst the three chemicals, which is:

ATP-Na being either greater than or approximately the same as Ach, which is greater than K. cit, which is greater than the control, can be attributed, it is believed that the intensity of the main artery to the removal of the myocardium muscle also participates in this.

In other words, the time required from the shut-off of the main artery to the removal of the myocardium muscle was short in the case of K. cit as compared

with ATP-Na and Ach, with a result that it is believed that the extent of oxygen debt is small.

In the case of ATP-Na being either greater than Ach or approximately equal to Ach, is it true that the cardiac arresting action of Ach is superior to that of ATP-Na? Not necessarily. In the example using Ach, a cardiac arrest was obtained after an injection and, even if heart pulsation might appear again by internal operation, the myocardium was cut off as it was in conformity with the passage of time. Thus, ATP-Na being either greater than Ach or being approximately equal to Ach does not indicate the intensity of the cardiac arresting action. It is also believed that the amount of oxygen contained in the blood in the artery at the time of a partial extracorporeal circulation also participates in this. In other words, it can be stated that the oxygen debt of the myocardium becomes smaller when the amount of oxygen contained in the blood of the artery becomes greater.

In any case, there is hardly any obstacle to respiratory enzyme at the time of a cardiac arrest and it does not constitute a main factor in the irreversible stoppage of muscular contraction as stated by Bisg (5).

(6) Change in the Activity of ChE.

According to Burn (10), the relationship between auricular activity and Ach concentration is as shown in Figure 18. The regular range for the auricular activity and the concentration of Ach is between A and B. Between these two, if the concentration of Ach is raised by giving Ach, auricular activity comes down. When the concentration of Ach in the tissue is lower than A and when auricular activity is low as in the case of C, if the concentration is raised by giving Ach, the auricular activity becomes large and functions stimulatively.

(Insert Figure 18 on p. 1077. Relationship between auricular activity and Ach concentration (according to Burn).)

According to Alles and Hawes (1), further, the relationship between the concentration of Ach and ChE activity is as shown in Figure 19. When the concentration of Ach is raised, the hydrolytic action of the ChE becomes greater.

When a certain level of concentration is passed, it is reported that the action of ChE becomes lower. This result is in support of the aforementioned theory of Burn and makes it easier to understand the two opposing actions of Ach as far as the heart is concerned.

(Insert Figure 19 on p. 1077. Relationship between Activity of ChE and the Concentration of Ach (according to Alles and Hawes). a. Ach amount (millimol).)

In the example involving Ach, the lowering of the activity of Ch E amounted to 1.5 to 2.0 times those of the other two. This is believed to be due to the fact that the injection agent was Ach and there was greater enzyme activity for the decomposition of the injected Ach than in other cases, with a result that its activity was lost.

Even in the example of Ach where no perfect cardiac arrest was obtained, however, there was observed a lowering of the activity of ChE. Thus, it is not possible to discuss the cardiac arresting action solely from the activity of ChE. The details of its function and mechanism will have to be left for future examination.

Chapter 10: General Conclusion

Experiments on the elective cardiac arrest by using ATP-Na, ATP-K, Ach and K. cit under extracorporeal circulation by using an artificial heart-lung apparatus were carried out and, relative to the myocardium at the time of the cardiac arrest, the changes in the sugar (carbohydrate) metabolism, the amount of ATP, tissue respiration and the activity of ChE were examined. As a result of these examinations, the following general conclusions were obtained:

(1) In all cases, the amount of glycogen is once reduced four minutes afterward but is increased eight minutes afterward.

(2) In the examples where a fixed pattern was shown, there was a tendency for pyro grape acid to be reduced and for lactic acid to be once increased but to be reduced later.

(3) In the case of a cardiac arrest by ATP, the amount of ATP increased eight minutes afterward.

(4) The results described in (1) and (3) can be considered indicative of the possibility of the electrive cardiac arresting method as an increase in the amount of a power-source substance.

(5) In the examples involving the development of ventricular fibrillation, there was observed a reduction both in ATP and glycogen. The ventricular fibrillation should be avoided from the standpoint of energy metabolism.

(6) The activities of the succinic acid oxidation enzymes are all greater than the control. Their order was as follows:

ATP-Na being greater than Ach, which is greater than K cit. which is greater than the control.

This is considered to be an oxygen debt of the myocardium due to the shut-off of the main artery. It is indicative of an almost complete absence of an obstacle in the respiratory enzyme.

(7) In all cases, the activity of ChE is lower than that of the control. The order is as follows:

Control is greater than K. cit, which is greater than ATP-Na, which is greater than Ach.

This is indicative of a common process in the three chemicals in the cardiac arresting action.

(8) Under the assumption that the change in enzyme activity should be smaller than that of the control, the order of the supriority of the three chemicals including the intensity of the cardiac arrest action was determined. It was found to be as follows:

(I) K. cit, (II) ATP, (III) Ach.

The above-described order may change if other elements such as ventricular fibrillation and tissue changes received by the myocardium, etc. are taken into consideration.

At the end, the author would like to offer his deep thanks to Professors Hashimoto and Fukukei who offered constant guidance and encouragement and the joint researchers and to Mr. Yuichi Oi of the Department of Biology School of Science, University of Nagoya, who offered his self-sacrificing efforts.

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Table 1

	a	b		c	d	e
5	15	9		ATPase		+
6	12	9		同 上	47秒	−
7	13	9		同 上	49秒	−
8	14	9		同 上		−
16	12	9		ATPase	35秒	−

Table 2

	a	b		c	d	e
1	12	8			30秒	+
2	9	9		同 上	22秒	+
3	9	9		同 上	26秒	+
4	14	8		同 上	45秒	−
9	11	8		同 上		−

Table 3

	a	b		c	d	e
10	11	8				−
11	11	8		同 上		−
12	13	8				−

Table 4

	a	b		c	d	e
13	11				15秒	−
14	13	8		同 上	45秒	−
15	11	8		同 上	15秒	−

Table 5

		b	c	d	e	f	g	h
a								
ATPase	7							
	8							
	16							
Ach	10							
	11							
	12							
Kcl	13							
	14							
	15							

Table 6

		b	c	d	e	f	g	h	i
a									
ATPase	7								
	8								
	16								
Ach	10								
	11								
	12								
Kcl	13								
	14								
	15								

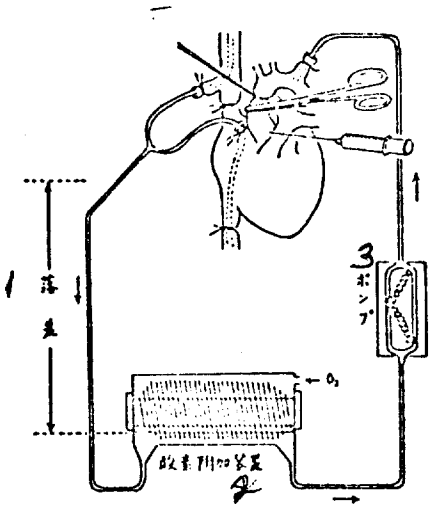
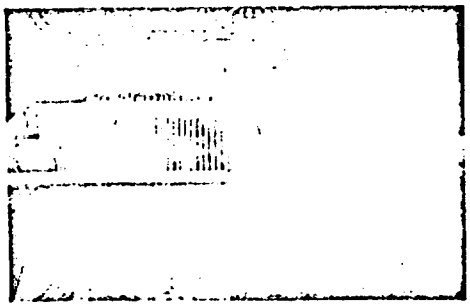


Figure 1



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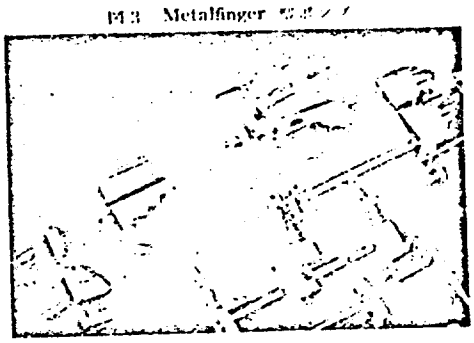
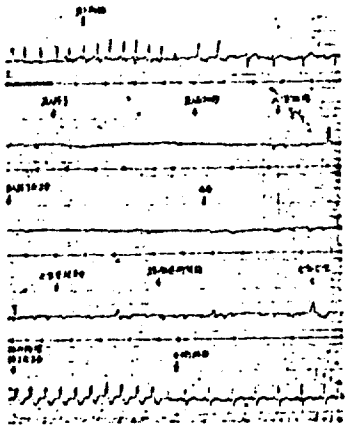


Figure 3



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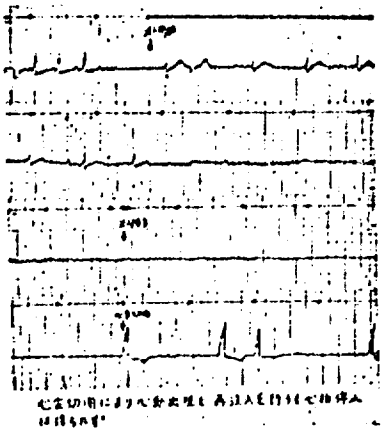

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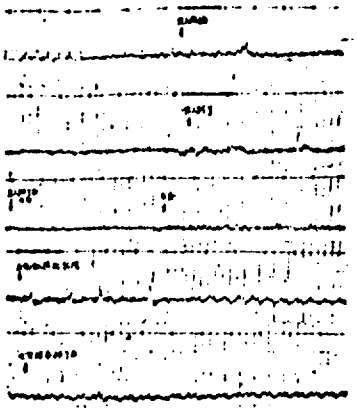

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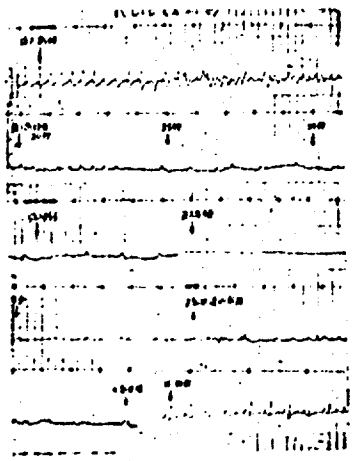

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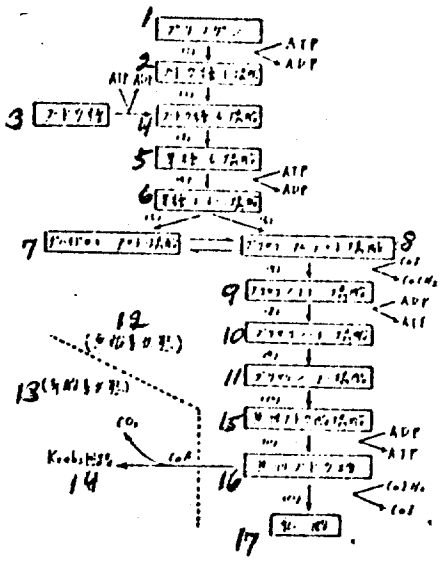


Figure 8

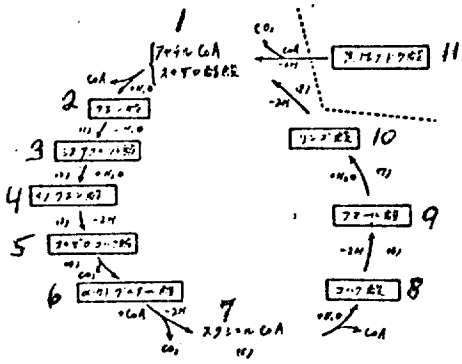


Figure 9

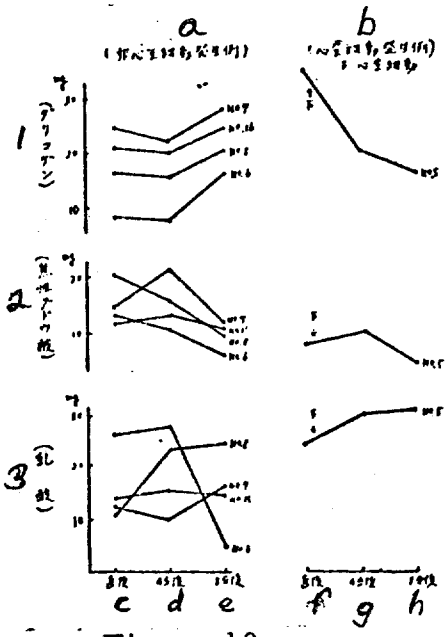


Figure 10

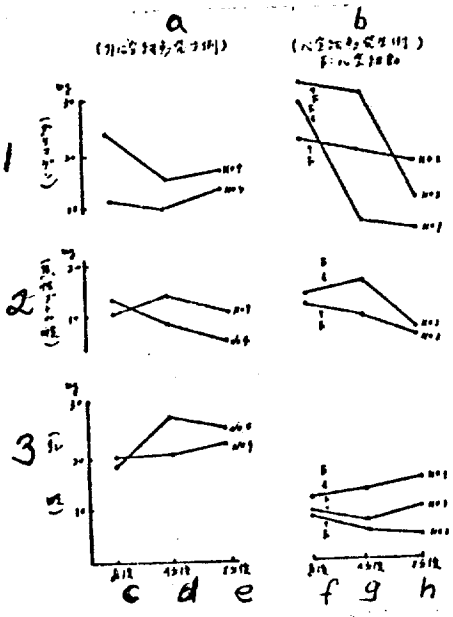


Figure 11

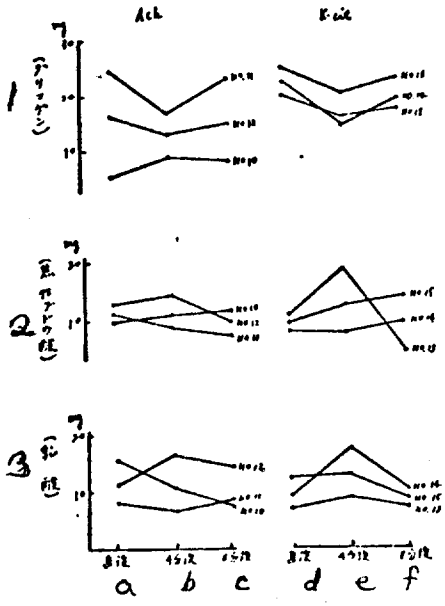


Figure 12

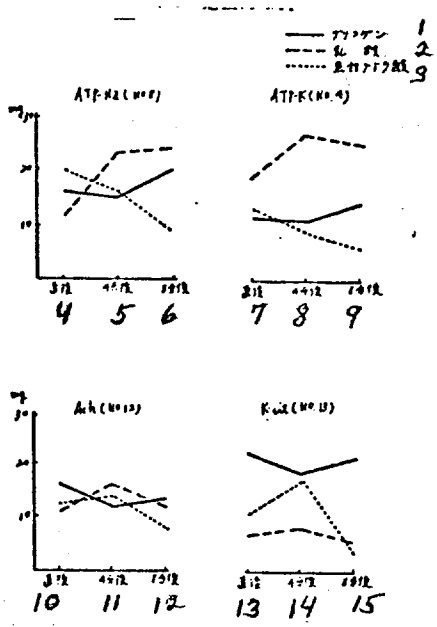


Figure 13

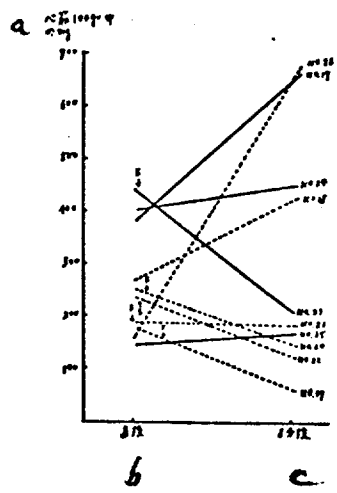
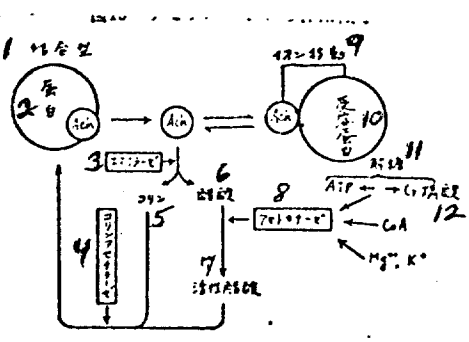


Figure 14



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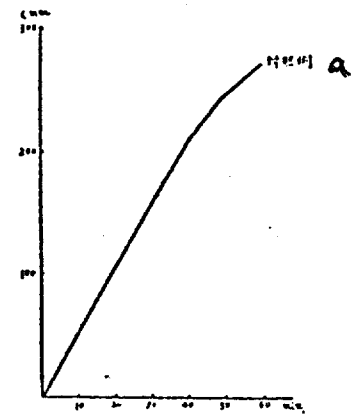

Figure 16

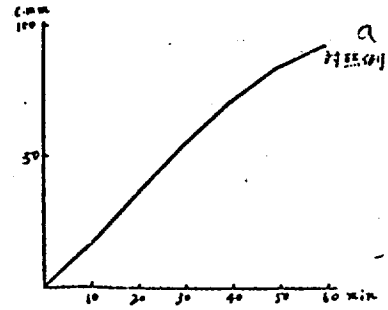

Figure 17

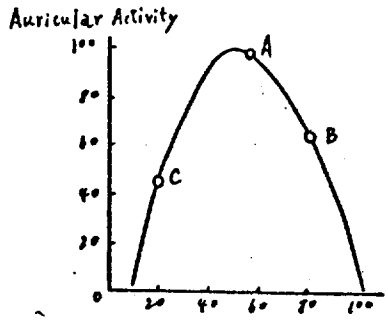

Figure 18

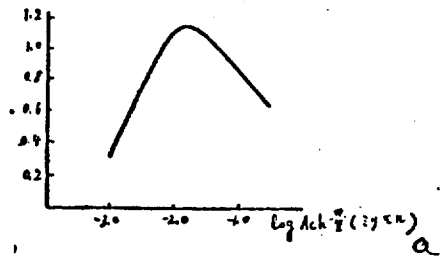

Figure 19

534

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アイカ・ベネヂクト型レスピロメーター

PAT. No. 378840
慶大内科笹本准助教授指導

- 9立スパイロメーター（ソーダライム罐付）
- ロールペーパー使用（連続描記可能）
- 2速度可変キモグラフ
- 1/25 吸気行程記録器
- 肺容積区分・MBC・時間肺活量の測定
- 酸素消費量　分時換気量の測定
- 肺疾患治療法の価値判断
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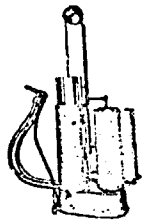

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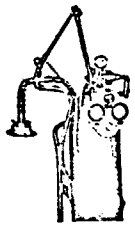

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慶大外科佐藤先生
慶大内科笹本先生　指導

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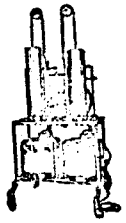

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心拍停止剤による心拍停止時の心筋糖代謝，組織呼吸及びコリンエステラーゼ活性度に関する研究

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（昭和34年10月7日受付）

本論文の要旨は次の学会及び協議会において発表した．昭和33年9月，第11回日本胸部外科学会．昭和33年11月，第2回心臓内直視下手術研究班協議会．昭和34年3月，第59回日本外科学会．昭和34年6月日本胸部外科学会第2回関西地方会．

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第1章　緒　言

直視下心臓内手術にあたつて，心臓切開中もなお継続する心拍動は，手術操作の困難，空気栓塞の発生，冠帰還血の大量喪失及び心筋のhypoxiaの問題等の難点を惹起する為，従来種々薬剤による任意心拍停止法が試みられて来た．

即ち，Björk[9] は1948年にアセチルコリン（以下 Ach と記す）を用いて，Swan[21] は1954年に Lam[17] は1955年にカリウムを用いてそれぞれ心拍停止を試み，遂に1956年，Kolf & Effler[12] はクエン酸カリ（以下 K. cit と記す）を用いて，Lam[18] は Ach を用いてその臨床応用に成功した．

一方，我々はアデノシン三燐酸（以下ATPと記す）による調節低血圧時の心電図所見で，R-R間隔の延長即ち徐脈が常に見られることから，これを任意心拍停止に応用しようとして，1957年以来そのナトリウム塩（以下 ATP-Na と記す）及びカリウム塩（以下 ATP-K と記す）について実験検討して来た[21]．

そこで人工心肺装置による体外循環下，ATP Na, ATP-K, Ach 及び K. cit による任意心拍

K Cit. 534

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停止実験を行い，心拍停止時における心筋糖代謝，組織呼吸及びコリンエステラーゼ（以下ChEと記す）活性度の変化などから3薬剤を比較してみた．又我々が主として行ったATP-Na，ATP-Kについてのみ心筋ATP量の変化を追った．

第2章　文献的考察

近年生化学の発達に伴い，物質代謝の研究はエネルギー代謝の研究へと移行し，これに伴い心筋代謝についても数々の知見が明らかにされつゝある．

心筋代謝を研究するには冠静脈カテーテル法，心筋標本，組織切片及びホモヂネートを用いる方法等がとられるが，この中組織切片又はホモヂネートを用いる方法は，代謝物質の各過程における変化を知るにはよいが，全体としての代謝系を捉捉するにはその条件が多分に生理的範囲を逸脱する欠点がある．心筋標本を用いても又この難はさけがたい．一方冠静脈カテーテル法によれば，心臓に出入りする物質の濃度は心筋代謝の和として知られるが，細胞内における変化，更には個々の酵素系の特質は明らかにされ得ない．しかしながら，後者は前者に比し心臓の代謝過程をより生理的条件下でつかみ得るという利点がある．以上の理由で，心筋代謝の研究は専ら冠静脈カテーテル法によって行われて来た感がある．

しかるに，心拍停止が行われた場合には冠血流が断たれているため，カテーテル法による代謝研究は心拍停止の或る時期にのみ限つて冠血流を行うような方法がとられ，又その内容も乳酸質を主とするものが多く，糖代謝に関するものは極めて少い．

Bentall[9] は K. cit による心拍停止実験で，一定時間の心拍停止冠血流を行い，血流中の乳酸量の変化から，

（1）拍動停止中の心筋代謝は乳酸生成にいたる場合解糖過程で保持されており，

（2）従つて，心筋には適当量のグリコゲンが貯えられていることが必要であり，この点，心拍停止に用いる K. cit 加血液にブドウ糖を加えることは力源物質の増量として意味がある，と述べている．これに関して Jordan[15] 等も K. cit の低張液としてブドウ糖の重要性をとりあげ，更に心拍再開にあたつて10%乳酸ソーダで冠血流を行い，直ちに effective な正常調律の心収縮が得られることから，カリウムの intoxication に対しては乳酸が有効であると述べている．

又，心筋組織呼吸についてみると，カテーテル法による実験結果から Bing[5] は次のような結論を下した．

（1）心拍停止後2時間まで心筋の酸素摂取量は徐々に増加する．それ故，呼吸酵素系が障害されていなければ，冠血流遮断時に心筋は酸素負債の状態になつていると考えられる．

（2）実際，心臓活動の不可逆的な停止は，心筋の収縮性蛋白質（アクトミオシン）の変性や呼吸酵素の死滅によるものではなく，正常刺戦伝導性及び調律の損失を伴つた筋線維細胞膜における変化によるのである．

Ach が刺戦伝導，筋収縮にさいして本質的な役割を演ずる物質 neurohormon であることは Nachmansohn[20] 以来あまねく認められるところである．心臓に対して，何故に Ach が相反した2作用（刺戦的及び抑制的）をもつかに関して，Burn[10] は家兎における実験から，組織内 Ach が正常以下の低濃度である時 Ach を与えると刺戦的に作用し，更に正常以上の高濃度になるように与えると抑制的に作用すると考えた．又 Ach 濃度とその分解酵素である ChE の活性度との間には，Ach の濃度を大にすると活性度も大となるが，或る濃度をこえると活性度は低下するという関係があるとの Alles & Hawes[1] の報告は，上述の Burn の見解を支持するものと考えられ，又こゝに Ach の臨床応用への道があるといえよう．

以上の考察からみて，ATP，Ach 及び K. cit の3者を比較するにあたり，糖代謝，組織呼吸及び ChE 活性度の変化を追うことは極めて有意義且つ興味あることと考えられる．又その方法には，生理的条件を逸脱するきらいはあるが，心筋組織自体又はホモヂネートを用いた．

第3章　実験方法（体外循環に関して）

実験には体重9～15kgの成犬を用い，全て静脈麻酔後気管内挿管を行い，以後はエーテルによつて麻酔を維持した．体外循環には円板状螺旋型酸素附加装置（名大W C型）（図1，2）及び metalfinger 型ポンプ（図1，3）を使用した．吸引用カニューレは右総腕静脈及び右心耳より上大静脈に，送血用カニューレは左鎖骨下動脈に挿入した．吸引は40～60cmの落差によつた．部分体外循環を行つて十分バランスをとつた後，上下大静脈を遮断し，大動脈を大動脈弁の末梢側でテープ及び大動脈鉗子によつて2回に遮断する．ついで心拍停止剤を表1～4に示す如く溶いて，急速に左

図1　体外循環下心拍停止剤注入を示す

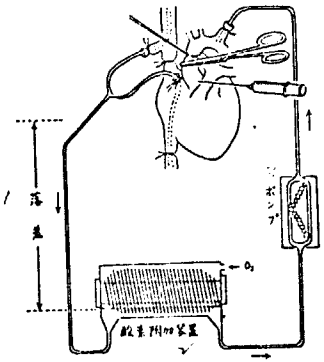


図2　酸素附加装置（名大W C型）

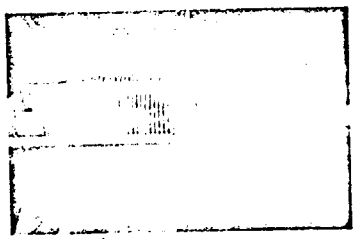
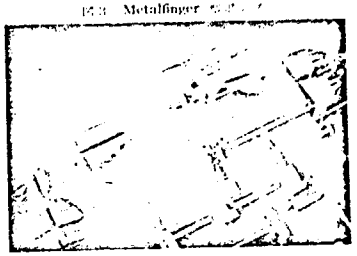


図3　Metalfinger



心室上部又は大動脈起始部に注入する．注入開始後，心拍動は次第に緩徐となり，遂に拍動停止状態となる．そこで右心室切開を加え，拍動停止直後，4分後及び8分後の3回にわたつて右心室壁心筋を切除し定量に附した．

正常拍動の再開は大動脈の遮断解除によつて得られる．

第4章　実験成績

糖代謝，ATP量の変化，組織呼吸及び ChE 活性度の変化に関しては別に章を設ける．

第1節　心拍停止状況

ATP-Na による5例では，心拍停止までの時間は35～55秒で，その中2例においては有効的に拍動停止状態でありながら心室壁の収縮減を認めた（表1）．

ATP-K による5例では，心拍停止までの時間は20秒～1分20秒であり，大動脈の遮断が不十分であつたと考えられる1例を除いて，ATP-Na に比し短かかつた．これはATPとカリウムの相乗作用の為と考えられる（表2）．

Ach による3例の中，No. 10では注入開始後

表1　ATP-Na による場合

5	15	9	ATP-Na 溶液 20cc 注入	40秒で拍動停止	40秒で拍動停止	+
6	12	9	同	上	47秒	−
7	13	9	同	上	45秒	−
8	14	9	同（注入2回）	上	2回目注入後の方が35秒で拍動停止	−
16	12	9	ATP-Na 溶液 1cc 注入		35秒	−

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表2　ATP-K による場合

No.	体重 kg	性	注入薬剤及び注入量	心拍停止までの時間	心室細動
1	12	♂	ATP-K 500mg を 5% 糖水 100cc に溶解して	30秒	+
2	9	♀	同　上	22秒	+
3	9	♀	同　上	32秒	+
4	14	♂	同　上 (注入2回).	2回目注入後約 45秒	−
9	11	♂	同　上	1分30秒で自然に洞調復(心動停止状態)	−

表3　Ach による場合

No.	体重 kg	性	注入薬剤及び注入量	心拍停止までの時間	心室細動
10	11	♂	塩化アセチルコリン 0.3g を 20cc の生食に溶して	10秒で心拍停止するが注入後 5~10分間で徐脈が起こし 再注入すると心拍停止せず	−
11	11	♂	同　上	心拍停止するも 完全には停止せず	−
12	13	♂	塩化アセチルコリン 0.3g を 20cc の生食に溶して 注入の際	7秒で停止するも心室は Mb の徐脈のため心拍停止は不明瞭	−

表4　K cit による場合

No.	体重 kg	性	注入薬剤及び注入量	心拍停止までの時間	心室細動
13	11	♂	注入薬剤は 注入量	15秒	−
14	13	♂	同　上 内容量 10cc	45秒	−
15	11	♀	同　上 内容量 13 5cc	15秒	−

10秒で心拍動は停止したが，心室切開等の操作を加える度に収縮がみられ，No. 11では極度の徐脈がみられたが完全には停止せず，No. 12では注入開始後7秒で心拍動は停止したが，心室切開により拍動出現し，再注入によっても拍動停止は得られなかった（表3）．

K. cit による3例では，心拍停止までの時間は5～15秒であった（表4）．

なお心拍停止の方法，心室細動及び薬剤によっておこる心筋の組織学的変化等に関しては，共同研究者丁村が詳細に追究しているので省略する．

第2節　心電図

ATP，Ach 及び K-cit の3者のいずれの場合にもほゞ臨床的所見に一致して心電図上でも収縮波は消失したが，ATP-Na による場合にのみ，

Fig 4　図4　心電図（心筋切除を行わない例）

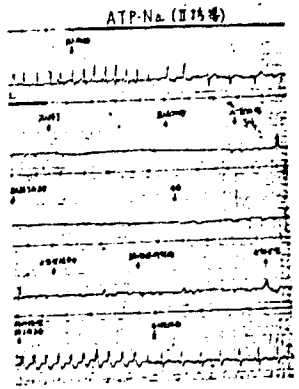
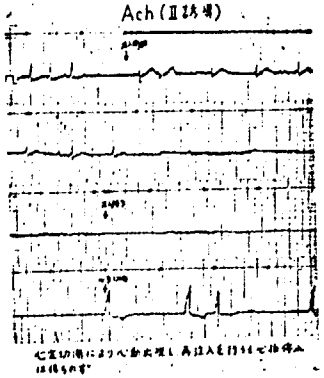


Fig 5　図5　心電図（心筋切除を行わない例）



心室切開によりペ動出現し再注入を行はずに心停止は得られず

内服的には拍動停止状態でありながら収縮波を認めた例があった．又拍動停止後にみられたP波を伴わぬ不整収縮波は手術操作によるものと考えられる（図4，5，6，7）．

第5章　糖代謝

第1節　心筋糖代謝のあらまし

心筋は Purkinje 氏線維及びミトコンドリアが豊富に存在する点で，その形態学的構造におい

Fig 6　図6　心電図（心筋切除を行わない例）

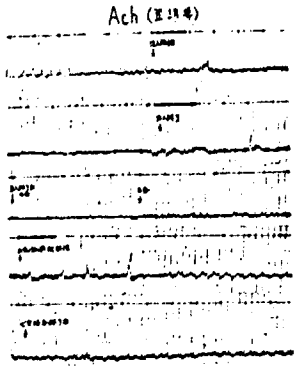
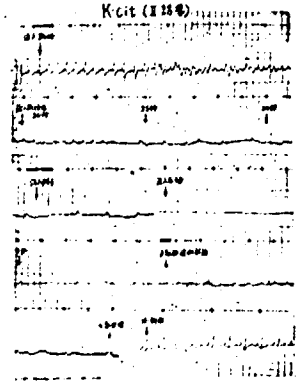


Fig 7　図7　心電図（心筋切除を行わない例）



て他の骨格筋とやゝ異なるが，代謝機転からみるとほゞ同じ基本的代謝過程を営んでいると考えられる．但し，血液中の含水炭素，脂肪酸及びアミノ酸等を摂取し，又組織自身の中間代謝産物を酸化分解して，これと同時に燐酸化反応が共軛してエネルギーの生成，貯蔵又は合成反応を行い，適当な平衡を保ちながら機能を営んでいる．そしてこれらの分解と合成の過程には極めて多数の酵素が関与している．

心筋が仕事をするためのエネルギー源には，ブドウ糖，乳酸，焦性ブドウ酸，酪酸，ケト酸及び脂肪酸などが挙げられるが[7]，そのエネルギー発生の主体をなすのは解糖過程である．グリコゲンがブドウ糖－6－燐酸をへて焦性ブドウ酸，更に乳酸にまで代謝される過程はすべて嫌気的に行われ嫌気的解糖といわれる．この経路に属する反応は可逆的とされている．本経路で生ずる焦性ブドウ酸は好気的条件で Krebs 回路[8]に入り完全に炭酸ガスと水に分解されるが，ここまで完全に酸化されるとブドウ糖1分子当り710,000 cal を生ずるのであるが，嫌気的解糖過程では完全酸化の約1/12 但し 57000cal を発生するにすぎない．しかしながらこの過程は

(1)　酸素の供給なしに行われ，しかも非常に速かに進行すること，従って生体内の急速なエネルギーの需要に応ずるのに適当な場合である．

(2)　生じたエネルギーが非常に高率（約65%）にATPとして蓄積され利用される．

という特質がある．

嫌気的解糖過程を示すと図8の如くである．反応（4）但し果糖－6－燐酸から果糖－1－6－

Fig 8　図8　嫌気的解糖過程（酵素名省略）

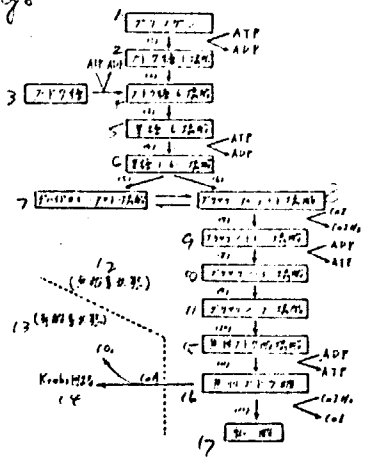


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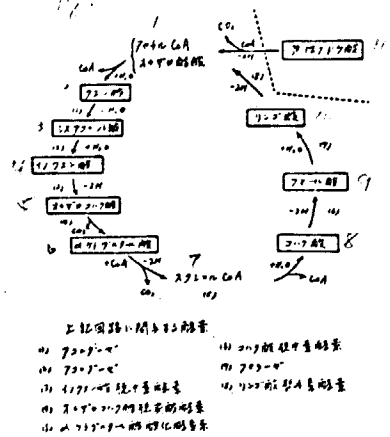
図9　Krebs 回路



二燐酸への逆反応は特別の燐酸酵素の作用の下に行われる．又反応（12）により生じた乳酸は燃焼されなかぎり拡散し，血液によつて肝に運ばれ，こゝで解糖経路の逆コースをへてブドウ糖又はグリコゲンに合成される．乳酸は好気的条件でそのまゝ酸化されることなく，必ず一旦焦性ブドウ酸に再酸化されて後酸化過程に入る．グリコゲンに合成される場合にも，一旦焦性ブドウ酸になつてから再合成される．従つて，乳酸は糖代謝の中間代謝産物ではなく，1つの最終産物又は安定な産物とみなされる[33]．

糖質の終末的酸化分解過程（Krebs 回路）を示すと図9の如くで，これは又脂質，蛋白質にも共通の過程である．

先ず焦性ブドウ酸は次の反応によりアセチル CoA の形の活性酢酸になる．

$CH_3{\cdot}CO{\cdot}COOH + DPN + CoA \rightarrow$ アセチル$CoA + CO_2 + DPNH_2$ 活性酢酸は CoA・ATP によりオキザロ酢酸のエノール型と反応してクエン酸を生じ Krebs 回路に入る．クエン酸はアコニダーゼの作用によつて水を失つてシス・アコニット酸となり，更にこれはアコニダーゼの作用に水を附加してイソ・クエン酸になる．更にイソ・クエン酸脱水素酵素により脱水素されてオキザロコハク酸を生ずる．次にオキザロコハク酸脱水炭酸酵素により脱炭酸されて α-ケトグルタール酸になる．更に α-ケトグルタール酸酸化酵素系によりコハク酸となり，コハク酸はコハク酸脱水素酵素によりフマール酸になり，ついでフマラーゼの存在で水を添加してリンゴ酸になる．リンゴ酸はリンゴ酸脱水素酵素により脱水素されてオキザロ酢酸になり，オキザロ酢酸は自発的異性化によりエノール化して再び回路に入る．

以上が Krebs 回路のあらましであるが，このさいの基質の酸化に共軛して燐酸化反応が行われ，ATP の高エネルギー燐酸結合の解明と共に，生体における呼吸エネルギーを捕捉する系として重要視されている．

そこで，心拍停止時における糖代謝をうかがうてだてとして，嫌気的解糖過程でのグリコゲン，焦性ブドウ酸及び乳酸を定量し，又組織呼吸をうかがう為，コハク酸酸化酵素（コハク酸脱水素酵素及びチトクローム酸化酵素）の活性度の変化を追つた（第6章で詳述）．

第2節　定量材料及び定量法

心拍停止直後，4分後及び8分後の3回にわたつて切除した右心室壁心筋は，脂肪組織及び結合組織部をさけ，又濾紙で十分血液成分をぬぐい去り，正確にその1gr を秤量してグリコゲン定量用に供した．乳酸及び焦性ブドウ酸の定量には，心筋1gr を10cc の三塩化酢酸でホモヂネートとしたので，その1cc を乾燥重量測定用に供し，乾燥重量 200mg で表わした．

定量は，グリコゲンはヂフェニールアミン法[9]，焦性ブドウ酸は Lu & Nelson 氏法[10][26]，乳酸は Barker & Summerson 氏法[3][27]により，又比色は Beckmann 光電比色計によつた．

第3節　定量成績

第1項　ATP-Na による場合

図10に示す如くで，心室細動を発生しなかつた例では，グリコゲンは4分後では殆ど変化がないか又はわずかに減少し，8分後に増加する．焦性

図10　ATP-Na による場合

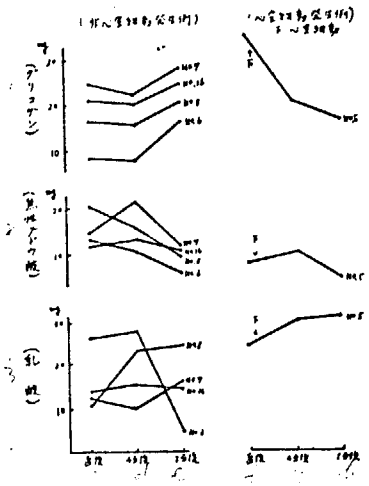
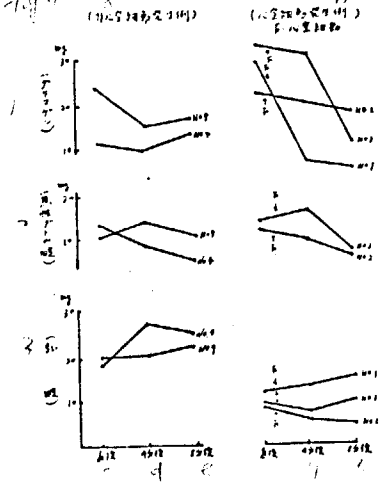


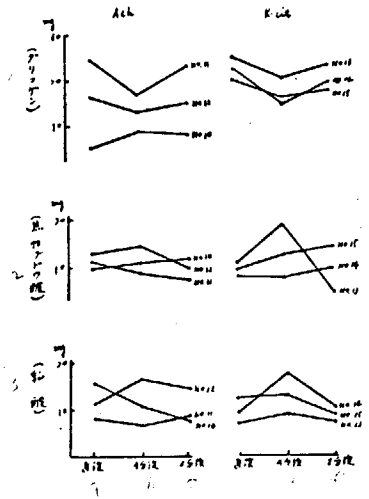
図11　ATP-K による場合



ブドウ酸には1例を除いて減少の傾向がみられた．乳酸には一定の傾向を見出し難い．

心室細動を発生した例では，グリコゲンは著明に減少し，焦性ブドウ酸は一旦増量して後減少し，乳酸は増量した．

図12　Ach, K, cit による場合



第2項　ATP-K による場合

図11に示す如くで，心室細動を発生しなかつた例では，グリコゲンは一旦減少して後増加し ATP-Na による場合と同様である．焦性ブドウ酸及び乳酸には一定の傾向を見出し難い．心室細動発生例ではグリコゲンは著明に減少し，焦性ブドウ酸及び乳酸には一定の傾向を見出し難い．3例中2例に乳酸の減少をみたのは注意に値する．

第3項　Ach による場合

図12に示す如くで，3例とも心室細動を発生せず，グリコゲンは2例は一旦減少して後増加し，1例は一旦増加して後減少した．焦性ブドウ酸及び乳酸には一定の傾向を見出し難い．

第4項　K. cit による場合

図12に示す如くで，3例とも心室細動を発生せず，グリコゲンは一旦減少して後増加し，焦性ブドウ酸には一定の傾向がみられぬが，乳酸は一旦増加して後減少した．

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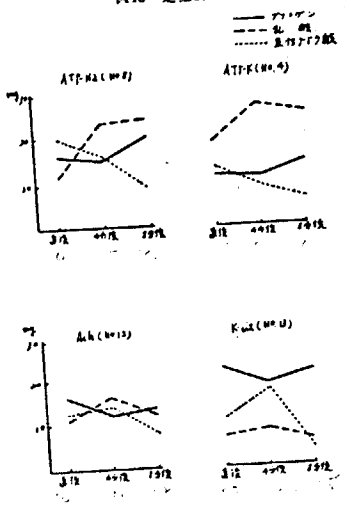
図13　定型的な例



第4節　小　括

ATP-Na, ATP-K, Ach 及び K. cit による心拍停止実験で，心筋グリコゲンについてのみ4分後に一旦減少して8分後に増加するという一定の傾向がみられたが，焦性ブドウ酸及び乳酸にはグリコゲンにおけるが如き一定の傾向を見出し得なかつた．しかしながら，定型的な経過をとつた実験例での定量値を4者について示すと図13の如くであつて，その傾向はほゞ同一であるといえよう．

第6章　ATP 量の変化

第1節　生体における ATP の意義

生物が生活している時，その生命の維持と生長に必要な諸物質の合成，筋収縮のような機械的な仕事，物質の吸収や排泄の際の滲透的な仕事，神経系統や発電器官における電気発生など，これら生理的諸過程に必要とされるエネルギーの窮極的な源泉は，高エネルギー燐酸結合，即ちATPの燐酸結合に貯わえられているエネルギーと考えられている．

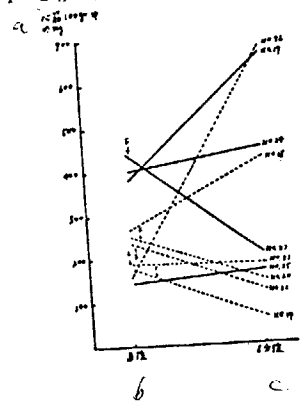
その1g分子につき1コの燐酸基が加水分解される時約 12000cal のエネルギーが放出される．従つて，生活を維持していく上にはエネルギー消失過程でATPの燐酸基消失により生じたADPから，何らかの仕方でATPが再生産され補充されなければならない．このATPの合成は，例えば嫌気的解糖過程で2分子のATPを生じ，Krebs回路で30分子のATPを生ずるとされているが，なおその生成反応の不明な点が残されている．

近年，Szent-Györgyi[25]一派によりアクトミオシンとATPの相互作用に関する研究がすゝみ，筋収縮におけるアクトミオシン，ATP及び塩類の間の反応機構である contraction cycle が考えられるにいたつた．しかしながら，筋収縮にさいし，ATP分子内の化学的エネルギーが如何にして機械的エネルギーに転換されるか，筋肉内に存在する収縮性物質の本態は何かなどに関しては，未だ決定的な理論がつくりあげられていない現状である．いずれにせよ，筋収縮にさいしATPが重要な役割を演ずることは論をまたない．

そこで，我々が主として行つた ATP-Na 及び ATP-K による心拍停止時において，その直後と8分後の心筋ATP量の変化を追つてみた．

第2節　定量材料及び定量法

図14　ATP量の変化（心拍停止はATPによる）
F：心室細動　－－ATP-Na　　ATP-K



心拍停止直後と8分後の2回にわたり切除した右心室壁心筋を用いた．

定量には，Cohn and Carter[11] によりはじめて adenosine polyphosphate の分析に使用せられたイオン交換樹脂によるカラムクロマトグラフィーを用いた．これは多数存在する定量法[28]の中，最近特に著しく発達し且つ筋肉のadenosine polyphosphate の定量に適しているといわれ，その中，宮崎，内田等によつて改変された方法によつた[30][32]．その装置，抽出法，試薬，実験操作及び計算式の詳細は省略する．

第3節　定量成績

図14に示す如くで，心室細動発生例を除いては，ATP-Na, ATP-K いずれの場合にも直後に比し8分後に20～30%の増量を示した．心室細動発生例ではすべて減少した．

第4節　小　括

ATPの増量は，これがその生産過程である嫌気的解糖過程，組織呼吸に関連するのであらためて考按の部でふれることとする．心室細動発生例では，ATPがエネルギー発生にあずかり減少したと考えられる．

第7章　組織呼吸

第1節　組織呼吸とエネルギー代謝

嫌気的解糖は生体における基本的力源過程の1つであるが，遊離酸素が与えられると，生体は呼吸というより有効な手段でエネルギーを獲得する．このことは前述の嫌気的解糖過程で2分子のATPを生じ，Krebs 回路で30分子のATPを生ずることからも明らかである．組織の代謝と密接な関係があると見なされている呼吸酵素の中，コハク酸脱水素酵素による酸素吸収反応にはチトクローム酸化酵素が関係し，その態度は全く生細胞の呼吸と平行関係にあると考えられている．

そこで，心拍停止時についてその組織呼吸をうかがう為，コハク酸酸化酵素の活性度の変化を Warburg 検圧装置を用いて追つた．

第2節　実験材料

心拍停止直後及び8分後の2回にわたつて切除した右心室壁心筋は，脂肪組織及び結合組織部をさけ，又濾紙で十分血液成分をぬぐい去り，正確にその1gr を秤量した．ついで 0.1M KCl でホモヂネートとなし，その 0.5ccを検圧用に，別に1ccを乾燥重量測定用に供した．なおホモヂナイズの操作は可及的速かに行い，又氷中に保つて酵素の破壊されるのを防いだ．

第3節　実験装置

装置は検圧計，振盪装置及び恒温槽の3部より成り，振盪は検圧計の軸を中心に回転運動を行う．検圧計は容器と Barcroft 氏 manometer の2部より成り，容器は円錐状で恒数 1.0～1.3 のものを用いた．容器恒数は藤田氏検圧法[31]のきめ方により，閉塞液は Brodie 液を使用した．

第4節　実験方法及び試薬の調製

実験は藤田氏の方法を参照し，主として Umbreit の記載する方法に従つて行つた[22][27]．反応系の組成は次の如くである．

0.1 M PO_4 (pH 7.4)	1.0cc
0.5 M Na-Succinate (pH 7.4)	0.3cc
Cytochrome C	0.4cc
4×10^{-3}M $CaCl_2$	0.3cc
心筋ホモヂネート	0.5cc
H_2O	全量を3ccとする

恒温槽の温度は37℃とし，振盪回数は1分間100～120回，ガス腔は空気とし，中央調室には2 NKOH 0.2cc宛を入れて呼吸により発生する炭酸ガスを吸収せしめた．又試験用検圧計の他に温度気圧計として対照用検圧計を用い，ガス腔内の温度及び気圧の変化によつておこる試験用検圧計の読みの誤差を補正した．そして恒温，恒容の条件下で酸素吸収によりおこる圧の減少を検圧計で読み，所定の式により吸収された酸素量 (c. mm) を算出した．

Warburg は組織呼吸を比較するにあたつて

$$QO_2=\frac{\text{酸素吸収量 (c. mm)}}{\text{組織乾燥重量 (mg)}\times\text{時間 (hr)}}$$

を用いているが，我々の実験では条件が一定しているので QO_2 に換算せず，反応がほゞ直線的に進む（図15）40分における酸素吸収量を以て比較することとした．

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図15　コハク酸酸化酵素 O_2 uptake 曲線
(c.mm/20mg dry weight)

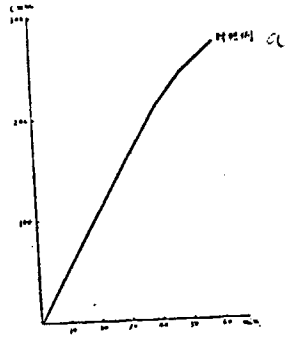


Table 5
表5　コハク酸酸化酵素 O_2 uptake
(c.mm/20mg dry weight)（自動酸化分控除値）

対照								
ATP-Na	7	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
		[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	8	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
		[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	16	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
		[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Ach	10	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
		[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	11	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
		[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	14	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
		[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
K. cit	13	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
		[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	14	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
		[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	15	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
		[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

第5節　実験成績

対照として，正常犬3頭を用い挿管麻酔下に拍動臍能にある右心室壁心筋を切除し，その酸素吸収量を測定した．個々の例での対照に対する変化を百分率で示すと表5の如くである．

ATP-Na による例では直後で＋22%～＋33%，8分後で＋29%～＋41%であつた．

Ach による例では直後で＋5%～＋34%，8分後で＋11%～＋42%であつた．

K. cit による例では直後で 0～＋18%，8分後で＋13～＋24%であつた．

第6節　小　括

ATP-Na，Ach 及び K. cit のいずれの場合にも心筋コハク酸酸化酵素の活性度は対照より大であつた．その順序は

ATP-Na'，Ach＞K. cit＞対照

であつた．又直後の値に比し8分後の方が活性度が大であつた．

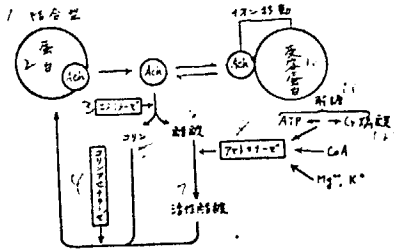
第8章　コリンエステラーゼ活性度の変化

第1節　コリンエステラーゼの意義

心臓が活動する時には刺激伝導，筋収縮に伴い活動電流を発生するが，このような生物電気現象は Ach の生成，消滅に関係する[14]．

Ach の生成とその受容蛋白との結合に基ずく原形質膜の変化によつてイオンの移動を生じ生物電気が起るものと理解されている[20]．

図16　アセチルコリンの作用機序



Nachmansohn によれば，図16に示す如く，結合型の Ach は作用はないが刺激を受けるとこの Ach が遊離する．この遊離した Ach が原形質膜を構成する受容蛋白に結合し，膜の構造を変化させ，従つてナトリウムイオンが移動をはじめる．そして Ach は直ちに ChE によつてコリンと酢酸に分解される．更に生じたコリンと酢酸はコリンアセチラーゼにより再合成されるが，この回復過程には解糖過程で生じたクレアチン燐酸がATPをへてエネルギーを供給する．

そこで，ATP，Ach 及び K. cit による心拍停止時に，Ach の分解酵素である ChE の活性度の変化を Warburg 検圧装置を用いて追つた．

第2節　実験方法

ChE の活性度を測定するには生物学的方法，化学的滴定法など種々あるが，最もしばしば用いられるのはガス測定による方法である．1933年，Ammon が Warburg 検圧装置を用いて行つた方法に[2]，その後多数の研究者により改良が加えられたが，本実験では Glick の記載する方法[13]によつた．

心拍停止直後，8分後の2回にわたり切除した心筋は5ccの Ringer液でホモヂネートとなし，その 0.5ccを検圧用に，別に1ccを乾燥重量測定用に供した．

原理：コリンのエステルから分解遊離する酸によつて，重炭酸ソーダから発生する炭酸ガスを測定する．

重炭酸リンゲル液組成（pH 7.4）

0.9 %	NaCl	100cc
1.26%	$NaHCO_3$	30cc
1.76%	$MgCl_2 \cdot 6H_2O$	2cc

この液は使用前に95% N_2，5% CO_2 混合ガスで飽和せしめる．主室には上記重炭酸リンゲル液1cc及び心筋ホモヂネート 0.5ccを，Sidearm には 1/10 M塩化アセチルコリン 0.5ccを入れ総量を 2.0ccとした．振盪回数は1分間90～100回，恒温槽の温度は37.5℃とした．

図17　コリンエステラーゼ CO_2 output 曲線
(c.mm/20mg dry weight)

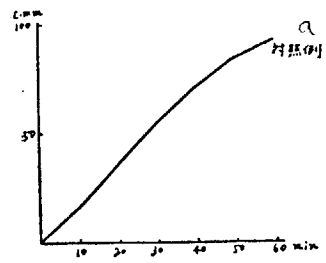


表6　コリンエステラーゼ CO_2 output
(c.mm/20mg dry weight)

対照										
ATP-Na	7	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	
		[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]		[illegible]
	8	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	
		[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]		[illegible]
	16	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	
		[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]		[illegible]
Ach	10	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	
		[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]		[illegible]
	11	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	
		[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]		[illegible]
	12	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	
		[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]		[illegible]
K. cit	13	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	
		[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]		[illegible]
	14	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	
		[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]		[illegible]
	15	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	
		[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]		[illegible]

第3節　実験成績

第6章におけると同様に対照例3頭の右心室壁心筋の CO_2 output を求めた．その曲線は60分までほゞ直線的にすゝむので（図17），60分における output CO_2 量（cmm）を以て比較することとした．その結果は表6の如くである．

ATP-Na による3例では約－40%で，直後に比し8分後にやゝ回復の傾向がうかがわれる．

Ach による3例では直後で約－60%，8分後で約－50%で，直後に比し8分後にみられる回復の傾向は3薬剤の中最も強い．

K. cit による3例では直後で約－25%であり，之に比し8分後にやゝ回復の傾向がうかがわれる．

第4節　小　括

ATP-Na，Ach 及び K. cit による心拍停止時において，いずれの場合にも ChE の活性度は対照より低下し，その順序は

対照＞K. cit＞ATP-Na＞Ach

であつた．

第9章　考　按

我々の行つた実験では，大動脈が遮断されてい

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る為，動脈血を介しての心筋への諸物質や酸素の供給はない．この条件下で実験成績に検討を加えてみる．

（1）グリコゲンの変化

筋に刺激を与えて収縮させるか，筋を無酸素状態におくかするとグリコゲンが減少して乳酸が蓄積するが，この筋を空気中に放置するか酸素を与えて休息させると，呼吸が行われて蓄積した乳酸は減少しグリコゲンが増加する．この時実際にみられる乳酸の消失量を，酸素吸収量から計算した乳酸の消失量と比較すると後者は前者の1/6程度にすぎないことから，Meyerhof は乳酸が呼吸によつて遊離されたエネルギーにより再びグリコゲンに再合成されると解釈した．彼の説明によれば，生細胞内の仕事に利用されるエネルギーは解糖作用によつて発生するのであつて，この解糖作用は酸素の有無にかゝわりなくおこり，たゞ酸素がある時には乳酸がグリコゲンに合成されて蓄積しないから，乳酸生成が阻害された結果になるというのであつた．この有酸素状態における醗酵の抑制現象，即ち Pasteur 効果に関してその後数々の研究が加えられ，その説明には多数の説が公表されているが，どれか1つの説によつて首尾一貫した説明をするのは困難のようである[29)30)]．

4分後におけるグリコゲンの減少は，心拍停止前の心拍動に由来する glycolysis の延長とも考えられるが，8分後におけるグリコゲンの増量に Meyerhof の解釈をあてはめるには，有酸素状態という条件が十分みたされない．即ち，心拍停止後心筋に与えられる酸素としては，心筋内に存在する動脈血からと，心室切開後心筋の表在部分が空気にふれるだけで，その酸素量は極めて少いと考えられる．又8分後においてグリコゲンの増量がみられたにもかゝわらず乳酸の増量の認められた例があつた．これらの理由から，嫌気的解糖の逆過程によるグリコゲンの増量という解釈は全面的にうけいれ難い．又既存心筋内のブドウ糖からの合成も考えられる過程の1つである．いずれにせよ，心拍再開を前にしてのこのグリコゲンの増量は，力源物質の増量として極めて有利な条件の一つと見なし得る．

心室細動発生例におけるグリコゲンの減少は，心筋の高度のエネルギー需要に応じて分解し減少したと考えられる．

（2）焦性ブドウ酸の変化

生体内にあつて，同一条件下では焦性ブドウ酸は乳酸とほゞ同様の変化の傾向をたどるとされているが，我々の実験ではかゝる傾向はみられなかつた．全体的には焦性ブドウ酸には減少の傾向がみられたが，後述の乳酸増量の傾向と考え合わすと，酸素不足の状態で乳酸に移行したか，或はその一部が Krebs 回路に入り分解したとも考えられる．しかしながら，焦性ブドウ酸は複雑な内的諸条件下にある不安定な中間代謝産物であるので，グリコゲンや乳酸におけるが如き意義を見出すことは困難である．

（3）乳酸の変化

乳酸には一旦増量して後減少又は増量の傾向がみられたが，これは解糖の結果として理解される．心室細動発生例においては，予期に反して著明な乳酸の増量をみず減少した例さえあつた（図11）．このことは，effective な心収縮を得るのに乳酸が有効であるとの Jordan の見解[16)]を裏がきするものと考えられ，他面グリコゲンの増量のないことから，エネルギー源として Krebs 回路に入り分解したとも考えられる．しかしながら，心室細動の結果乳酸が減少したのであるか，乳酸の減少が心室細動発生の1因子をなすのであるか，その因果関係は明らかでない．

（4）ＡＴＰ量の変化

ATP-Na, ATP-K による心拍停止例では，心室細動発生例を除いて，直後に比し8分後に著明なＡＴＰの増加をみたが，これはグリコゲンの増量と共に力源物質の増量として意義がある．この場合，注入剤が定量されるＡＴＰと同一物質である為，注入したＡＴＰが測定値に含まれる結果にならぬかという問題を生ずるが，ＡＴＰ自身非常に不安定な物質であり，定量操作に入るまで注入されたＡＴＰがそのまゝとゞまるとは考えられず，又注入したＡＴＰが定量値に含まれるとすれば，8分後の値に比し直後の値が高くてしかるべきである．以上の理由で測定されたＡＴＰは心筋固有のものと考えてよいであろう．

心室細動発生例でのＡＴＰの減少は，これがエネルギー発生にあづかり消費されたものと考えられる．抗心室細動作用 antifibrillatory action を有するＡＴＰ[23)]が何故に心室細動を発生せしめるかは，体温など他の要素を合わせ検討しなければならないであろう．

以上糖代謝を中心に ATP，Ach 及び K. cit の3者を比較すると，3薬剤の間には著しい差異は認められず，特に心拍再開前におけるグリコゲン及びＡＴＰの増量はエネルギー代謝の面から任意心拍停止法の可能性を示すものといえよう．又心室細動発生例では，グリコゲン及びＡＴＰが減少し，更に乳酸にさえ減少のみられるところがあり，これら力源物質の減少することから心室細動を回避すべきことは論をまたない．

（5）組織呼吸

ATP-Na，Ach 及び K. cit による心拍停止時において，組織呼吸と平行関係にあると考えられている心筋コハク酸酸化酵素の活性度は，いずれの場合にも対照より増強の傾向を示した．そして直後に比し8分後の方が活性度の強いことから，Bing のいう如く，冠血流遮断にもとずく酸素負債と考えるのが至当であろう．このことは又心拍停止時においては，その解糖過程が嫌気的であるという Bentall[4)] の報ずるところを裏がきすると共に，呼吸酵素系の障害の殆どないことを示している．3薬剤の間の差異，即ち

ATP-Na≧Ach>K. cit>対照

なる活性度の差異が如何なる本質的な薬理作用にもとずくかは明らかでないが，その心拍停止作用の強さ，従つて大動脈遮断から心筋切除までの時間も関係すると考えられる．即ち，K. cit は ATP-Na，Ach よりも大動脈遮断から心筋切除までの時間が短かく，従つて酸素負債の程度が低いと考えられる．又 ATP-Na≧Ach では Ach の方が ATP-Na よりも心拍停止作用が優れているかというそうではなく，Ach による例では，注入後心拍停止が得られ，心内操作によつて心拍動が出現しても，そのまゝ時間的推移にしたがつて心筋を切除したので ATP-Na≧Ach は心拍停止作用の強さを示すものではない．又部分体外循環時の動脈血の酸素含有量も関係すると考えられる．即ち，動脈血の酸素含有量の大なる方が心筋の酸素負債の程度が低いといえよう．

いずれにせよ，心拍停止時において呼吸酵素の障害は殆どなく，筋収縮の不可逆的停止において主たる原因をなすものではないとは Bisg[5)] の述べるところである．

（6）ChE 活性度の変化

Burn[10)]によれば auricular activity と Ach 濃度との関係は図18の如くである．auricular activity と Ach 濃度の正常範囲は A-B 間で，この範囲の状態にある時 Ach を与えて組織内 Ach の濃度を大にすると auricular activity は低下し，又組織内の Ach 濃度が A より低く auricular activity も低い例えはCである時，

図18　Auricular Activity と Ach 濃度との関係（Burn による）

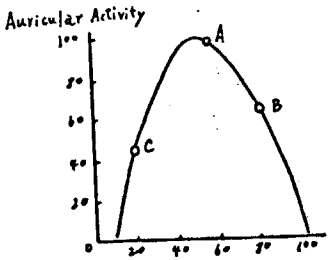
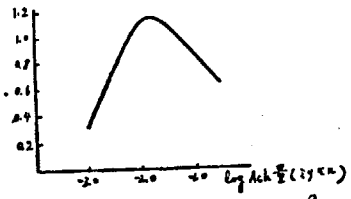


図19　ChE 活性度と Ach 濃度との関係（Alles & Hawes による）



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Ach を与えて濃度を大にすると auricular activity は大となり刺激的に作用するというのである。

又 Alles & Hawes[1] によれば，Ach 濃度と ChE 活性度との関係は図19の如くである．Ach の濃度を大にしていくと ChE の加水分解作用は大となるが，或る濃度をこえると ChE の作用は低下するというのである．この成績は上述の Burn の見解を支持するものであり，心臓に対する Ach の相反した2作用を容易に理解せしめる。

Ach による例では，ChE 活性度の低下が他の2者の 1.5～2.0 倍に及んだが，これは注入剤が Ach であり，他の場合よりも注入した Ach を分解するだけより多く酵素活動をなし，その活性度を失つたものと考えられる。

しかしながら，完全な心拍停止の得られなかつた Ach による例でも ChE 活性度の低下を認めたので，ChE 活性度のみで心拍停止作用を論ずることは出来ず，その作用機転の詳細は今後の薬理学的問題として残される。

第10章　結　論

人工心肺装置による体外循環下，ATP-Na, ATP-K, Ach 及び K. cit による任意心拍停止実験を行い，心拍停止時の心筋についてその糖代謝，ATP量，組織呼吸及び ChE の活性度の変化を追い次の結論を得た。

(1)　グリコゲンはいずれの場合にも4分後に一旦減少し，8分後に増加する。

(2)　定型的な経過をとつた例では，焦性ブドウ酸には減少，乳酸には一旦増加して後減少する傾向がみられた。

(3)　ATPによる心拍停止例では，8分後にATPは増加する。

(4)　(1),(3)の結果は力源物質の増量として任意心拍停止法の可能性を示すものといえる。

(5)　心室細動発生例ではグリコゲン，ATPともに減少し，エネルギー代謝の面からは心室細動は回避すべきである。

(6)　コハク酸酸化酵素の活性度は対照に比しいずれも大であり，その順序は

ATP-Na＞Ach＞K. cit＞対照

であつた．これは大動脈遮断による心筋の酸素負債と考えられ，呼吸酵素系の障害の殆どないことを示している。

(7)　ChE の活性度は対照に比しいずれの場合にも低下し，その順序は

対照＞K. cit＞ATP-Na＞Ach

であつた．これは心拍停止作用における3薬剤の共通過程を示すともいえる。

(8)　酵素活性度の変化は対照に比し少い方が望ましいという前提の下に，心拍停止作用の強さを含めて3薬剤の優劣の順序を定めると（Ⅰ）K. cit,（Ⅱ）ATP,（Ⅲ）Ach となる．心室細動，心筋の受ける組織学的変化など他の要素を考え合わせるとこの順序はかわるかも知れない。

調筆するにあたり，終始御懇篤なる御指導をたまわつた橋本教授，福富助教授並びに共同研究者各位，更に献身的な協力を惜しまれなかつた名古屋大学理学部生物学教室大井俊一氏に深甚の謝意を表する。

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AN EXPERIMENTAL STUDY ON CARBOHYDRATE METABOLISM, TISSUE RESPIRATION AND ACTIVITY OF CHOLINESTERASE OF THE MYOCARDIUM DURING ELECTIVE CARDIAC ARREST INDUCED BY CARDIOPLEGIC AGENTS

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In order to investigate the carbohydrate metabolism, the contents of grycogen, pyruvic acid and lactic acid were determined immediately 4 and 8 minutes after cardiac arrest, under extracorporeal circulation using a heart-lung apparatus. Activities of succinoxidase and cholinesterase were searched by the Warburg manometer. ATP content of the myocardium was traced only in the cases of elective cardiac arrest by ATP.

1) Carbohydrate metabolism

In carbohydrate metabolism there was no significant difference between the results of the three agents in the satisfactorily progressed cases. Glycogen was not markedly

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altered or was slightly decreased 4 minutes after cardiac arrest, and it was increased 8 minutes after cardiac arrest. Pyruvic acid was decreased after a period of increase, or showed consistent decrease. Lactic acid was decreased after a period of increase, or showed consistent increase. In the cases in which ventricular fibrillation had taken place remarkable decrease in glycogen content was noted, but in the pyruric acid and lactic acid content there was no significant changes.

2) Changes in ATP content

In the cases without ventricular fibrillation during cardiac arrest by ATP, ATP content was increased 8 minutes after the onset of arrest.

In the cases with ventricular fibrillation ATP content was remarkably decreased.

3) The activity of succinoxidase

With all three agents used, activity of succinoxidase was elevated compared with the control according to the following order:

ATP>Acetylcholine>K-cit>Control.

These findings are considered to be due to the oxygen debt of the myocardium caused by aortic occlusion.

4) The activity of cholinesterase

With all three agents used, activity of cholinesterase was depressed compared with the control according to the following order:

Control>K-cit>ATP>Acetylcholine.

This depression of cholinesterase activity may be considered to be the common process of the three agents through their cardioplegic actions.

With the concept that in the process of cardiac arrest less alteration of the enzymic activity is desirable, and also supported by the result of the experiment on the carbohydrate metabolism, the author gives the order of suitability of the three agents as follows:

1) K-cit 2) ATP 3) Acetylcholine.

(The author's abstract)

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MYOCARDIAL NECROSIS FOLLOWING ELECTIVE CARDIAC ARREST INDUCED WITH POTASSIUM CITRATE

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ELECTIVE cardiac arrest, induced in the course of open cardiac operations with cardiopulmonary bypass, has been found to be a valuable technical adjunct. In particular, the quiet and dry field it provides greatly facilitates the repair of the more complex anomalies. Of the various methods available for producing elective asystole, the technique devised by Melrose[1] has been most widely employed. In this technique, the heart is arrested by the injection of a solution of potassium citrate into the ascending aorta, proximal to an occluding clamp. This method of arrest was often employed at the National Heart Institute and its advantages seemed clear. Sometimes, however, difficulty was encountered in re-establishing effective myocardial contraction and an occasional patient presented clear evidence of left ventricular failure in the post-arrest period. These observations suggested a physiologic evaluation of the effects of potassium citrate on myocardial function.[2] The hearts of 2 patients who died after potassium citrate arrest revealed, on routine histopathologic study, unusual areas of necrosis and prompted a detailed pathologic re-examination of the hearts of all patients who died following open cardiac operations. The results of these studies in 30 patients are described in the present report.

MATERIALS AND METHODS

Material available for the study consisted of the hearts of 30 patients who had died at various intervals following direct vision intracardiac operations. Nineteen patients had had elective cardiac arrest induced with potassium citrate. In none of the remaining 11 patients had this technique been employed; in 9 no form of arrest was used and arrest was induced in 2 by aortic occlusion alone. In every patient, total cardiopulmonary bypass was provided by either a modified Melrose pump-oxygenator (12 patients) or a rotating disc oxygenator with a nonocclusive roller pump (18 patients). Systemic flow was, in general, provided at a rate of 2 L. per minute per square meter of body surface area and the duration of perfusion varied from 9 to 113 minutes.

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Received for publication Sept. 8, 1959.

When elective arrest with potassium citrate was induced, a solution of 2.5 per cent potassium citrate was prepared by diluting a 25 per cent solution with nine volumes of fresh, heparinized blood. The aorta was cross-clamped immediately distal to the heart and the arresting solution was injected as rapidly as possible into the aortic segment until complete myocardial paralysis was observed. Following completion of the intracardiac procedure, coronary flow was restored by removal of the aortic clamp. In all instances the right heart was drained during the recovery period to prevent distention. The periods of aortic occlusion ranged from 6 to 67 minutes.

Routine pathologic examination of the hearts of these 30 patients had been made at the time of autopsy and they had been fixed in formalin and stored. They were re-examined grossly and full-thickness sections were prepared from blocks taken from the anterior wall, posterior wall, and apex of the left ventricle as well as from the ventricular septum and the wall of the right ventricle. Additional sections of both coronary arteries were also studied. Although these various sections were taken from pre-determined places in the ventricles, care was taken to avoid the surgical incisions or other obviously traumatized areas.

RESULTS

A distinctive type of necrosis was observed in various portions of the myocardium in the hearts of 15 (79 per cent) of the 19 patients subjected to potassium citrate arrest. The distribution and severity of the necrotic myocardial lesions, together with pertinent clinical data, are presented in Table I. This lesion was not observed in any of the hearts of the 11 patients who did not receive potassium citrate at the time of intracardiac surgery.

Characteristically the lesions consisted of multiple, sharply circumscribed, microscopic areas of necrosis which usually occurred in the central portion of the ventricular myocardium. Their appearance is shown, at various magnifications, in the representative photomicrographs reproduced in Figs. 1 through 5. The necrotic fibers were swollen and stained intensely with eosin. The cytoplasm in these fibers was either hyalinized or granular and neither cross striations nor longitudinal fibrils could be seen. The nuclei were large and stained strongly with hematoxylin. Even in the acute lesions, those observed in patients who died 2 hours or less after cardiac arrest, there was already disintegration and dissolution of portions of the necrotic fibers and granular debris could be seen between the larger, intensely stained fragments. A striking feature of the lesions was the absence of both hemorrhage and congestion. The cut surface of the myocardium in those hearts exhibiting the largest and most diffuse lesions was grossly dark and discolored. This darkened, mottled appearance was more pronounced in the central portions of the myocardium and often the subendocardial zone was quite pale. The over-all appearance followed closely the distribution of the lesions as shown in Figs. 1 and 2.

The amount of myocardial necrosis varied from single isolated microscopic areas observed in one histologic section, to massive necrosis, involving as much

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TABLE I. CLINICAL SUMMARIES AND PATHOLOGIC FINDINGS IN PATIENTS SUBJECTED TO CARDIAC ARREST WITH POTASSIUM CITRATE

PATIENT AND NUMBER	AGE (YR.)	SEX	DIAGNOSIS	DURATION OF CARDIOPULMONARY BYPASS (MIN.)	DURATION OF ARREST (MIN.)	VOLUME OF 2.5% K CITRATE (C.C.)	DURATION OF LIFE AFTER ARREST	DEGREE AND DISTRIBUTION OF MYOCARDIAL NECROSIS*			
								LVA	LVP	SEPTUM	RV
1. E. R. (01-59-24)	1½	M	Ventricular septal defect	20	10	50	4 hr.	0	0	0	+
2. D. V. (01-62-55)	25	F	Ventricular septal defect	34	25	40	5 da.	0	++	+	0
3. P. L. (01-53-77)	5	F	Atrial septal defect and ventricular septal defect	43	22	20	2 hr.	++	0	+	0
4. J. P. (01-74-93)	13	F	A-V canal	28	20	40	26 da.	0	0	0	0
5. W. G. (01-81-62)	42	M	A-V canal	33	20	60	16 hr.	+	++	++	+
6. L. M. (01-91-69)		M	Tetralogy of Fallot	75	30	50	None	0	0	+	0
7. N. A. (01-68-73)	6	F	Ventricular septal defect	18	6	60	28 hr.	0	0	0	0
8. H. W. (00-85-09)	22	M	Tetralogy of Fallot	31	24	60	48 hr.	0	0	0	0
9. J. K. (01-91-66)	4	F	Ventricular septal defect	26	15	20	1½ hr.	++	0	0	+
10. D. S. (00-80-73)	2	M	Ventricular septal defect	30	15	20	28 hr.	0	0	0	0
11. D. Y. (00-88-77)	4	F	Ventricular septal defect	26	13	20	24 hr.	+	0	0	0
12. M. P. (01-94-08)	6	M	Ventricular septal defect	26	20	60	16 hr.	++++	++++	++++	++
13. S. D. (00-93-72)	39	F	Congenital aortic stenosis	25	15	30	10 da.	++++	++++	++++	+
14. R. L. (00-99-66)	8	M	Ventricular septal defect	36	15	30	2 hr.	++++	++++	++++	0
15. A. W. (00-98-42)	7	M	Tetralogy of Fallot	46	15	*	12 hr.	+	0	+	0
16. E. B. (01-85-33)	18	M	Supravalvular aortic stenosis	113	67	80	None	+++	+++	+++	+
17. D. F. (01-83-98)	10	M	Congenital aortic stenosis	25	12	27	12 hr.	++++	++++	+++	+
18. L. B. (00-95-07)	8	M	Tetralogy of Fallot	59	24	30	16 hr.	0	++	0	++++
19. M. D. (02-25-37)	4	F	Tetralogy of Fallot	41	16	25	28 hr.	0	+++	0	+

*LVA = anterior wall of left ventricle; LVP = posterior wall of left ventricle; RV = right ventricle.

as 50 per cent of the myocardium, seen in several sections. These degrees of necrosis were designated as + to ++++ in the various sections examined and their distribution in the anterior wall of the left ventricle (LVA), posterior wall of left ventricle (LVP), ventricular septum and right ventricle (RV) is shown in Table I. The lesions were severe (+++ to ++++) in 6 patients, moderate (++) in 2 patients and mild (+) in 7 patients. No lesions were found in the hearts of 4 patients. It is evident from the data presented in Table I that the lesions were more severe in the left ventricle than in the right, although only one block from the right ventricle was examined.

Fig. 1.—Patient No. 14 (00-99-66) was an 8-year-old boy with ventricular septal defect who died 2 hours after potassium citrate arrest. Multiple, discrete areas of necrosis in the midportion of the wall of the left ventricle can be seen. Some of the lesions are confluent. Characteristically these lesions were neither hemorrhagic nor congested. (Hematoxylin and eosin; ×8; reduced ⅙.)

Sections of both the right and left coronary arteries were examined in each heart. The degree of atherosclerosis varied from patient to patient but did not appear to correlate well with either the severity or distribution of the myocardial lesions. Thrombosis or necrosis of coronary arteries was not observed to be associated with any of the lesions. The atria were not examined systematically but a portion of the left atrium was usually included in the block taken from the posterior wall of the left ventricle and necrosis was never observed in it.

In the second group of 11 patients, cardiopulmonary bypass had been employed at the time of intracardiac surgery but cardiac arrest was either not induced (9 patients) or was produced by aortic occlusion alone (2 patients). The anatomic defects necessitating operation and the conditions of perfusion were comparable to the patients listed in Table I. No necrotic myocardial lesions of the type just described were seen in sections of these hearts. Indeed, the only pathologic changes noted were those specifically associated either with the defect

for which operation had been performed, with the operative incisions themselves, or the small areas of acute hemorrhage and edema which follow cardiac massage and defibrillation.[3] In most instances, the myocardium appeared normal.

DISCUSSION

The patients are arranged in chronologic order according to the date of operation in Table I. It is apparent that the most severe lesions were encountered in Patients 12 to 19 and less severe lesions were seen in Patients 1 to 11. All 4 of the patients who received potassium citrate but whose hearts did

Fig. 2.—Patient No. 17 (01-83-98) was a 10-year-old boy with congenital aortic stenosis who died 12 hours after cardiac arrest with potassium citrate. The massive, confluent areas of necrosis involve more than half of the myocardium of the left ventricle. Note, as in Fig. 1, that the subendocardial and subepicardial layers of the myocardium tend to be spared. (Hematoxylin and eosin; ×100; reduced ⅙.)

not exhibit necrotic lesions were among the first 11 patients in the table. The date dividing these two groups was May, 1958, and re-examination of the surgical records shows that at this time a change was made in the pharmaceutical preparation of the 25 per cent potassium citrate stock solution. Before this date the solution was prepared in bulk and often had been stored in glass several months before it was used. However, precipitates were noted to form in the solution and flaking of the glass containers was observed. After May,

1958, the potassium citrate was furnished in crystalline form and the 25 per cent solution was prepared by adding a measured amount of diluent to a weighed quantity of the crystals immediately before use. Following this change in the procedure for the preparation of the potassium citrate solution, every patient who died after potassium arrest had myocardial necrosis. The lesion, in fact, was severe in the hearts of 7 of the 8 patients. It thus seems likely that the drug, when freshly prepared from crystals, resulted in a more toxic solution which was responsible for more severe myocardial damage.

The data presented do not allow a conclusive statement as to the pathogenesis of the myocardial lesions; they were, however, unequivocally associated with perfusion of the heart with potassium citrate. There was no correlation between the occurrence of the myocardial necrosis and the type of congenital defect, the heart size, age, sex, or any other clinical factor that was considered. It is also remarkable that the occurrence and severity of the myocardial lesions do not correlate well with the amount of potassium citrate infused into the coronary arteries, the length of perfusion, or the duration of cardiac arrest. The association of more severe myocardial damage with the change in the preparation of the potassium citrate solution also indicates that the myocardial lesions resulted from its use. Recent experimental work lends further support to this conclusion. Waldhausen and his co-workers[2] showed, in a comparison with acetylcholine and anoxia as arresting agents, that potassium citrate caused severe depression of left ventricular function. Gilbert[4] has demonstrated that the distribution of the potassium citrate solution in the myocardium is by no means uniform and this observation poses the problem as to whether the necrosis occurs in areas which have or have not received the highest concentration of potassium citrate.

After the present study was initiated, Helmsworth and his associates[5] described histologically similar lesions in the hearts of dogs sacrificed 48 hours after cardiac arrest had been induced with potassium citrate. The lesion has also been produced experimentally in the dog in our laboratory and is remarkably similar to the lesions observed in the human being (Fig. 6). This experimentally produced lesion is also remarkable because the profound pathologic changes in the myocardial cells occurred within 30 minutes after perfusion with potassium citrate. Fully developed lesions were also observed within 1 to 2 hours in several of the patients in the study. Older lesions in the patients, lesions observed in patients who died 2 days or longer after operation, exhibited infiltrates of leukocytes and macrophages but were otherwise quite similar histologically to the acute lesions. The myocardial lesions produced by the potassium citrate appear to be distinctive. Study of the fresh lesions in the dog heart suggest that segments of the myocardial fibers have undergone acute disintegration leaving swollen and intensely staining remnants which are widely separated. Capillaries, small arterioles, and connective tissues within the lesion appear to be unaffected. These various features distinguish this lesion from a variety of other types of myocardial necrosis such as occur in acute infarcts,[6] in association with potassium deficiency,[7] or with noradrenalin.[8]

A discussion of the clinical course of patients with widespread myocardial damage of the type described is difficult since in some it was altered or obscured

Fig. 3.—Same patient as in Figs. 1 and 4. This photomicrograph of a single lesion illustrates the discrete, sharp boundary between the area of necrosis and adjacent, normal myocardium. Many portions of the necrotic myocardial fibers have disintegrated and disappeared so that the remaining portions of the myocardial fibers appear widely separated. The thin-walled, intramural artery toward one end of the lesion has a normal appearance and its lumen is empty. The capillary-sized vessels are prominent because of the altered myocardium but they also appear normal and are free of thrombus. The lesion is not hemorrhagic. (Hematoxylin and eosin; ×100; reduced ⅙.)

Fig. 4.—Same patient as shown in Figs. 1 and 3. The portions of necrotic myocardial fibers which remain in the lesions are enlarged and stained intensely with eosin. The cell nuclei are enlarged and stained heavily with hematoxylin. Fragments of disintegrated fibers can be seen between the swollen fibers, but many portions of the injured fibers have dropped out. (Hematoxylin and eosin; ×275; reduced ⅙.)

by other significant factors such as postoperative hemorrhage or an incomplete anatomic repair. In others, however, death could clearly be attributed to the left ventricular failure which followed arrest. In these patients gradually

Fig. 5.—Patient No. 12 ((01-94-08) was a 6-year-old boy who died 16 hours after arrest with potassium citrate and repair of ventricular septal defect. The junction between normal appearing myocardial fibers and necrotic fibers is quite sharp. Fragmentation and dissolution of portions of necrotic fibers is apparent. (Hematoxylin and eosin; ×275; reduced ⅙.)

Fig. 6.—The histologic appearance of necrotic lesions in the dog heart after cardiac arrest with potassium citrate. The lesion is similar to that observed in patients. The animal was sacrificed 15 minutes after normal heartbeat was restored. (Hematoxylin and eosin; ×100; reduced ⅙.)

progressive hypotension was observed which became unresponsive to pressor agents, and was associated with rising venous pressure. One patient (No. 13) apparently survived this acute period of failure, only to die 10 days after operation of an unrelated cause.

The clinical and pathologic observations just described and the experimental demonstration of impaired ventricular function following potassium citrate arrest has led to the abandonment of this technique at the National Heart Institute. Although flaccid paralysis of the heart certainly facilitates the repair of many intracardiac anomalies, the hazard of the method is so great as to outweigh this advantage. A dry and quiet field can be secured by intermittent aortic occlusion alone and this technique is applicable in virtually all procedures in which aortotomy is not necessary. When the aorta must be opened for long periods, as in the repair of aortic valve lesions, direct coronary perfusion has been found the technique of choice.

SUMMARY

Detailed microscopic examinations were made of the hearts of 19 patients who died at various intervals following open cardiac operations in which cardiac arrest had been induced by the infusion of potassium citrate into the coronary bed. In 15 (79 per cent) of the 19 patients, myocardial necrosis of a characteristic type was demonstrated. The lesion was not observed in the hearts of 11 patients who underwent similar procedures without arrest or with arrest produced by aortic occlusion alone. The etiology of the necrosis and its significance in the choice of an operative technique for clinical use is discussed.

ADDENDUM

Since the manuscript was submitted, detailed histologic study of the hearts of 7 additional patients has been made. In 4, anoxic arrest was employed and, in 3, the heart was not arrested. No evidence of myocardial necrosis was found in any section.

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DISSECTING ANEURYSM OF THE THORACIC AORTA AS A COMPLICATION OF CATHETER AORTOGRAPHY: SUCCESSFUL SURGICAL TREATMENT

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The widespread use of translumber aortography which followed the original description of the method by Dos Santos and his colleagues[1] has resulted in numerous reports of complications of the procedure. Recent reviews of these complications have been written by Crawford and his co-workers[2] and McAfee and Willson.[3] In contrast, much less attention has been devoted to the complications of catheter aortography perhaps because this method has been applied much less often.

Abdominal aortography by injection through a catheter introduced by way of the femoral artery was first performed by Farinas.[4] Thoracic aortography by catheterization from the radial artery was described by Radner.[5] This method has been extensively applied with great success in Sweden.[6, 7] The sole complication in a large series of cases was a single mild cerebral injury which occurred following the injection of too high a concentration of opaque substance into the cerebral circulation as a result of incorrect placing of the tip of the catheter. Lane and Peirce[8] reported brachial plexus palsy following extravasation of 70 per cent Diodrast injected through a polythene catheter in an effort to demonstrate an innominate artery aneurysm. Deterling[9] reported the occurrence of cerebral complications presumably resulting from the dislodgment of an atheromatous plaque by a catheter introduced into the aorta from the left ulnar artery.

Dissection of the aorta during the performance of a catheter aortogram has not been reported but the occurrence of this complication with percutaneous translumber aortography is recognized. In an excellent discussion of this problem, Gaylis and Laws[10] presented 2 cases of their own with photographs demonstrating dissection of the abdominal aorta and discussed 5 cases in the literature which they considered similar. More recently, Fineberg and associates[11] reported the occurrence of gangrene of the large intestine and ovaries due to dissection of the abdominal aorta after translumber aortography. This

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Received for publication Oct. 12, 1959.

fallen far short of expectation. Nutritional problems as measured by inability to gain weight have been present in some degree in 48 to 60 per cent of the patients. The dumping syndrome although only mild and moderate has occurred in 46 per cent of the patients. It was hoped that gastroduodenostomy would be associated with fewer of these nutritional and dumping sequelae. Finally, gastroduodenostomy has failed in spite of adequate gastrectomy in the patient with resection for duodenal ulcer. Certainly 11 per cent recurrence, a rate that may increase as the follow-up is longer, is far more than obtained by conventional surgery. Table V compares our series with other published data. With the exception of Harkins and associates the recurrence rate in the duodenal ulcer group is disturbingly high. On the other hand in the gastric ulcer resections our recurrence rate was 0 and that in the comparative series is considerably lower than the duodenal ulcer group.

CONCLUSIONS

1. Two-thirds to three-quarters gastrectomy with gastroduodenostomy has been found to be associated with a significant percentage of postoperative nutritional and dumping problems.

2. The operation in spite of "anatomical adequacy" is a failure in patients with duodenal ulcer because of the high rate of recurrent ulcer.

3. The operation, up to follow-up reported, is not associated with recurrent ulcer in the gastric ulcer group, and continued clinical trial of the procedure for gastric ulcer is justified.

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AN EVALUATION OF VARIOUS CARDIOPLEGIC METHODS UTILIZING ISOLATED PERFUSED GUINEA PIG HEARTS

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Since its origination by Melrose, elective cardiac arrest has achieved a recognized place in open heart surgery. Some of the reasons advanced for this procedure are that the danger of air embolism is lessened, a motionless, almost bloodless field is provided with excellent visibility, blood loss is decreased, and, since the entire coronary circulation is stopped, there need be no fear of kinking or obstructing the coronary vessels. Open operations on the aortic valve would be impossible without stopping the heart unless the coronary arteries were separately perfused. Methods permitting arrest and ready reinstitution of the heart action should serve to extend further the scope of operative procedures that can be performed utilizing extracorporeal circulation techniques.

Successful elective cardiac arrest and recovery in isolated animal hearts, in intact animals, and in human surgery have been accomplished by Melrose and Effler using potassium citrate, Moulder and associates with acetylcholine; Young, Sealy, and associates utilizing potassium citrate and magnesium sulfate in combination; and Lam by injecting potassium chloride.

In a recent article Merritt and Sealy indicated that a combination of potassium, neostigmine, and magnesium is superior to potassium citrate alone. They state that although cardioplegia is immediate following the use of either of these methods, there is more rapid recovery on reperfusion following the use of the combined materials. Following the injection of potassium citrate alone, the heart required about 3 minutes of perfusion to return to a normal beat.

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Anoxia and profound hypothermia have also been observed to cause cardiac arrest, as has A.C.D. solution.[1]

The work herein reported was done to evaluate the effects of various cardioplegic agents on the isolated, perfused guinea pig heart. The actions of potassium citrate, acetylcholine, potassium citrate plus magnesium sulfate, A.C.D. solution, anoxia, and profound hypothermia were investigated and compared.

MATERIALS AND METHODS

Healthy, adult guinea pigs of either sex weighing from 500 to 800 grams were anesthetized with 30 milligrams per kilogram of sodium pentobarbital intraperitoneally. In most cases, a small amount of ether was required as a supplement. A tracheal cannula was inserted, artificial respiration instituted, and the chest opened. Heparin, 5 milligrams per kilogram, was injected into the inferior vena cava to prevent clotting. The pericardium was opened and the ascending aorta stripped of surrounding tissue and mobilized. A loop of heavy thread was placed around the aorta close to its origin and a second loop of No. 000 silk placed a few millimeters higher. The proximal loop of thread was then tightened, the aorta incised, and a glass cannula inserted into it. The tightening of the loop of thread prevented the cannula from passing through and damaging the aortic valves. After the distal thread was tied securing the cannula in place, the proximal loop was loosened. The great vessels were then severed and the heart was removed from the chest. The glass cannula was inserted into the per-

[1]A.C.D. solution, "Formula B," U.S.P. and N.I.H. Dextrose 1.47 grams, sodium citrate 1.32 grams, citric acid 0.44 gram per 100 cubic centimeters.

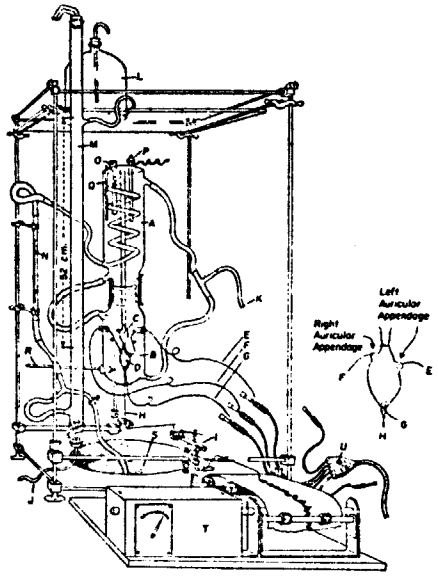

FIG. 1. The isolated perfused heart apparatus: *A*, constant temperature bath surrounding the warming spiral; *B*, double walled glass heart chamber; *C*, glass cannula; *D*, heart; *E*, *F*, *G*, electrodes placed as indicated in enlargement at right; *H*, loop of thread through apex of heart passed through an opening in outer glass chamber, around a pulley wheel and attached to writing lever *I*; *J*, inlet tube for 95 per cent oxygen and 5 per cent carbon dioxide; *K*, tube for attachment to compressed air to keep water circulating in constant temperature bath and double walled glass chamber; *L*, Mariotte bottle; *M*, oxygenator tube; *N*, flow meter; *O*, heating element with thermostat, *P*; *Q*, thermometer in water bath; *R*, thermometer through double walled glass chamber; *S*, funnel to catch and drain off the perfusion fluid flowing out of the heart; *T*, variable speed motor for turning recording paper on drum; *U*, electrocardiographic connector to electroencephalograph.

fusion apparatus and perfused according to the method of Langendorff as modified by Garb. The heart was subjected to a minimal period of hypoxia seldom exceeding 45 seconds from the time of removal to onset of perfusion. Hearts were perfused with Ringer-Locke solutions composed of: sodium chloride 7 grams per liter, potassium chloride 0.42 gram per liter, calcium chloride 0.24 gram per liter, magnesium chloride 0.2 gram per liter, or magnesium chloride hexahydrate 0.48 gram per liter. The perfusate was not recirculated. The perfusion fluid flowed downward through a tube 1 meter long interposed between a Mariotte bottle containing the Ringer-Locke solution and the warming spiral, while a gas mixture of 95 per cent oxygen and 5 per cent carbon dioxide in the form of tiny bubbles rose from a sintered glass disc at the bottom of the tube. Thorough oxygenation of the perfusion fluid proved essential for a well functioning preparation. In all experiments, perfusion pressure was 52 centimeters of water. A double walled glass chamber fitting about the heart was heated by several large light bulbs placed nearby. Temperature in the bath about the warming spiral was maintained by means of an electric heater controlled thermostatically. Simultaneous recordings of temperature of the bath and the chamber were taken.

A small loop of thread was passed through the apex of the heart, through an opening in the outer glass chamber, around a small pulley, and attached to a writing lever. Contractile force and heart rate could thus be measured directly from the tracings (Fig. 1).

After the heart had assumed a regular rate and rhythm and had reached maximum amplitude of contraction, the cardioplegic agents to be investigated were injected in such a way as to perfuse the coronary arteries. At the same time that the agent was injected, the perfusion fluid was stopped. After the desired period of arrest, the perfusion was resumed and allowed to continue until maximum recovery was achieved before additional tests were made. The order in which drugs were used was varied with each heart. All compounds were tested on at least 4 different hearts. With the exception of the hypothermic studies, all perfusions were done at a temperature of 38 degrees C.

Hypothermic conditions were achieved by using a refrigerant circulating about the perfusion spiral and through the double walled glass chamber. In these tests the perfusion fluid was not interrupted during the cooling or reheating period.

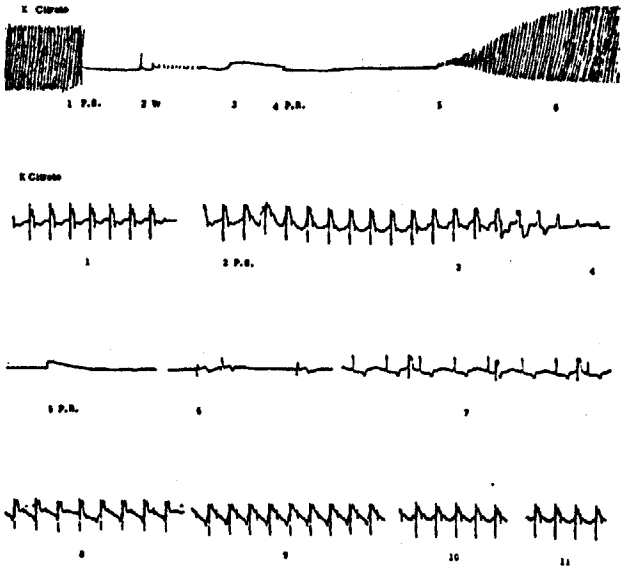

FIG. 2. Tracing of mechanical activity on upper line: *1 P.S.*, perfusion stopped and 1 cubic centimeter of 1.25 per cent potassium citrate injected followed by immediate arrest; *2 W*, solution washed through tubing with 0.6 cubic centimeter of Ringer's solution causing a few beats; *3*, return to mechanical arrest; *4 P.R.*, perfusion restarted after 60 minute arrest; *5*, onset of mechanical activity 45 seconds after perfusion restarted; *6*, return to maximum amplitude 90 seconds after reperfusion. Electrocardiographic tracings: *1*, control; *2 P.S.*, perfusion stopped and injection of 1 cubic centimeter of 1.25 per cent potassium citrate begun; *3*, injection completed; *4*, decreasing electrical activity which persisted for 2 seconds after mechanical arrest; *5 P.R.*, perfusion restarted after 10 minute arrest; *6*, onset of electrical activity 20 seconds after reperfusion; *7*, 1 minute; *8*, 2 minutes; *9*, 3½ minutes; *10*, 5 minutes; *11*, 8 minutes after perfusion reinstituted.

In the anoxic studies, the perfusion was stopped until mechanical arrest occurred.

The electrocardiograph was recorded by using 3 stainless steel wires as electrodes. Two of them, hook-shaped at the ends, were fixed to the right and left auricular appendix. The third one was placed through the apex of the heart. Lead II was recorded.

The materials used were obtained from commercial sources and consisted of (1) 1.25 per cent potassium citrate, given in 1 cubic centimeter amounts; (2) Sealy's mixture ($K_3C_6H_5O_7 \cdot H_2O$ 0.54 gm. and $MgSO_4 \cdot 7\,H_2O$ 2.7 gm. per 100 c.c. H_2O) brought to *pH* 7.4 with sodium bicarbonate, given in 2 cubic centimeter doses; (3) acetylcholine 2 cubic centimeters of 5 milligrams per cubic centimeter; (4) A.C.D. solution 5 cubic centimeters of 1:2 dilution of stock solution. All injections were made rapidly.

RESULTS (TABLES I AND II)

Potassium citrate. In 12 determinations using 1 cubic centimeter of 1.25 per cent potassium citrate injected into the coronary artery perfusion tubing, arrest was immediate. The average time for initial recovery was 43 seconds. Maximum recovery occurred on the average in 2 minutes 48 seconds. The average initial heart rate was 187 beats per

TABLE I.—MECHANICAL ACTIVITY

Agent	Initial (average) No. of determinations	Initial (average) Amplitude, cms.	Initial (average) Rate per min.	Time to arrest*	Duration of arrest	Time to recovery Initial, min.	Time to recovery Initial, sec.	Time to recovery Maximum, min.	Time to recovery Maximum, sec.	Maximum recovery Amplitude, per cent of initial	Maximum recovery Rate per min.
K citrate	12	1.9	187	Immediate	22 min.	0	43	2	48	104.8	187
Sealy's mixture	10	1.6	198	Immediate	19 min. 5 sec.	1	04	3	18	90.4	194
Acetylcholine	13	1.6	178	0.1 sec.	11 min. 8 sec.	0	22	5	10	93.2	174
A.C.D. solution	15	2.6	183	0.2 sec.	26 min.	1	31	3	16	87.1	196
Anoxia	10	2.0	192	5 min. 8 sec.	22 min. 1 sec.	0	47	5	51	82.4	205
Hypothermia	4	1.8	195	Temp. 16.2° C.	10 min.	Temp. 25.5°C.		Temp. 38°C.		78.2	176

*All times listed are averages.

TABLE II.—ELECTRICAL ACTIVITY

Agent	No. of determinations	Time to arrest*	Duration of arrest	Time to initial recovery	Time taken to return to regular rhythm	Remarks
K citrate	7	4 sec.	14 min. 20 sec.	24 sec.	1 min. 30 sec.	Although a regular rhythm occurred within 1½ min., control EKG pattern occurred on the average of 3 minutes after reperfusion was started
Sealy's mixture	7	6 sec.	12 min. 18 sec.	45 sec.	1 min. 46 sec.	Although a regular rhythm occurred within 1 min. 46 sec., control type of EKG pattern did not occur until average of 5 min. after reperfusion began
Acetylcholine	7	4 sec.	13 min. 24 sec.	20 sec.	1 min. 40 sec.	Although a regular rhythm occurred within 1 min. 40 sec., control type of EKG pattern did not occur until average of 5 min. after reperfusion began
A.C.D. solution	4	10 sec.	16 min.	35 sec.	2 min. 30 sec.	Although a regular rhythm occurred within 2 min. 30 sec., a control type of EKG pattern did not occur until average of 6 min. after reperfusion began
Anoxia	4	No electrical arrest attained to 70 min.	(Mechanical) 37 min. 30 sec.		4 min.	Upon oxygenation fibrillation began and persisted for 1-2 min. after which regular rhythm began. Control type of EKG pattern on average of 5 min. after reperfusion began
Hypothermia	2	Temp. 16.2° C.	10 min.	Temp. 23°C.	Temp. 38°C.	Regular EKG pattern began at temp. 30 C. and continued unchanged except for increase in rate until normal temp. reached

*All times listed are averages.

minute with an average amplitude of contraction of 1.9 centimeters. At the time of maximum recovery the average rate returned to 187 beats per minute. The average amplitude of contraction rose to 1.99 centimeters, slightly above the initial levels (Fig. 2). The periods of arrest varied from 5 minutes to 1 hour with an average time of 22 minutes. In 1 case (determination 13) a heart did not recover after 60 minutes of arrest. This preparation had undergone a 60 minute period of anoxic arrest 30 minutes earlier.

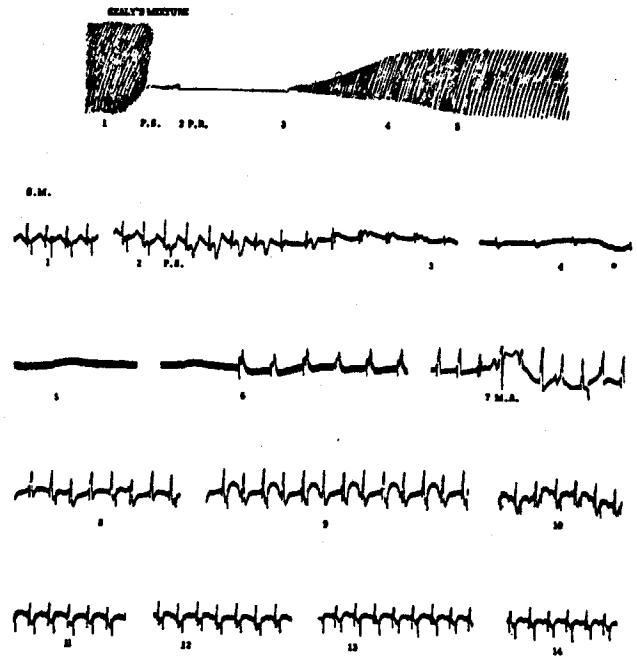

FIG. 3. Tracing of mechanical activity on upper line: *1*, control amplitude of contraction; *P.S.*, perfusion stopped and 2 cubic centimeters of Sealy's mixture injected followed by immediate arrest; *2 P.R.*, perfusion restarted after 30 minute arrest; *3*, onset of mechanical activity 30 seconds after reperfusion; *4*, amplitude of contraction increasing; *5*, maximum amplitude of contraction 100 seconds after restarting perfusion. At extreme right the tracing is at a higher speed of drum rotation to show the shape of the contraction wave. Electrocardiographic tracings: *1*, control; *2 P.S.*, perfusion stopped and injection of 2 cubic centimeters of Sealy's mixture begun; *3*, injection completed; *4*, decreasing electrical activity with arrest (indicated by asterisk) 4 seconds after injection ended; *5*, perfusion restarted after 10 minute arrest; *6*, onset of electrical activity 40 seconds after reperfusion; *7 M.A.*, mechanical activity began at this point 58 seconds after perfusion restarted; *8*, 2 minutes; *9*, 3 minutes; *10*, 5 minutes; *11*, 8 minutes; *12*, 10 minutes; *13*, 13½ minutes; *14*, 14 minutes after reperfusion.

Electrocardiographic determinations (Table II) done on 7 hearts arrested for an average of 14 minutes and 20 seconds each revealed that electrical activity persisted for 2 to 7 seconds, average 4 seconds, following mechanical arrest. Initial electrocardiographic recovery occurred on the average in 24 seconds with return to a regular rhythm in 1 minute 30 seconds (Fig. 2).

Sealy's mixture (potassium citrate and magnesium sulfate). A total of 10 determinations were done by injecting 2 cubic centimeters of this mixture into the coronary arteries through the perfusion tubing. Mechanical arrest was immediate in all instances. Initial recovery occurred on the average in 1 minute 4 seconds while maximum recovery averaged 3 minutes 18 seconds. The average initial heart rate was 198 beats per minute with an average amplitude of 1.6 centimeters. At the

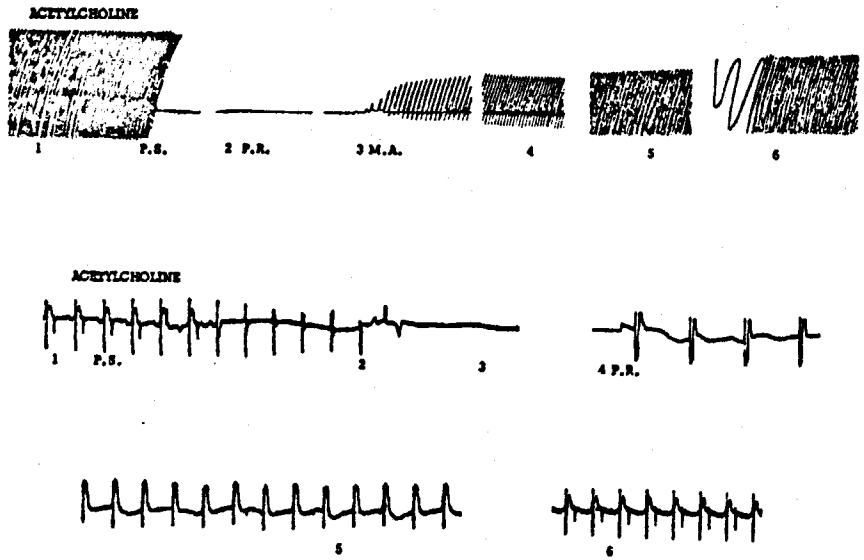

FIG. 4. Tracing of mechanical activity on upper line: *1*, control amplitude of contraction; *P.S.*, perfusion stopped 1.2 cubic centimeters of acetylcholine solution, 5 milligrams per cubic centimeter, injected followed by immediate arrest; *2 P.R.*, perfusion restarted after 30 minute arrest; *3 M.A.*, onset of mechanical activity 50 seconds after reperfusion; *4*, 2 minutes; *5*, 3½ minutes; *6*, 4 minutes after perfusion restarted. Electrocardiographic tracings: *1*, control; *P.S.*, perfusion stopped and injection of 2 cubic centimeters of acetylcholine chloride solution, 5 milligrams per cubic centimeter, begun; *2*, injection completed; *3*, arrest 1 second after injection; *4*, perfusion restarted after 10 minute arrest with rapid onset of electrical activity; *5*, 3 minutes; *6*, 11 minutes after reperfusion.

time of maximum recovery the average rate was 194 beats per minute. The average amplitude of contraction returned to 1.45 centimeters, 90.4 per cent of the initial value (Fig. 3). The periods of arrest varied from 9 to 30 minutes for an average of 19 minutes and 5 seconds.

Electrocardiographic determinations done on 7 hearts arrested for an average of 12 minutes and 18 seconds each revealed that electrical activity persisted for an average of 6 seconds after mechanical activity had ceased. Initial electrocardiographic recovery occurred on the average in 45 seconds with return to a regular rhythm in 1 minute and 46 seconds (Fig. 3).

Acetylcholine. In 13 determinations using 2 cubic centimeters of a 5 milligrams per cubic centimeter solution, mechanical arrest was immediate in 10 instances, took 0.8 second in 1 case, 1.0 second in another, and 0.6 second in a third for an average time to arrest of 0.1 second. Initial recovery occurred on the average in 22 seconds while the maximum recovery time averaged 5 minutes 10 seconds. The initial heart rate averaged 178 beats per minute with an average amplitude of contraction of 1.6 centimeters. At the time of maximum recovery the average rate was 174 beats per minute. The average amplitude of contraction returned to 1.49 centimeters, 93.2 per cent of the initial value (Fig. 4). The periods of arrest varied from 5 to 30 minutes for an average of 11 minutes 8 seconds.

Electrocardiographic determinations done on 7 hearts arrested for an average of 13 minutes and 24 seconds each revealed that

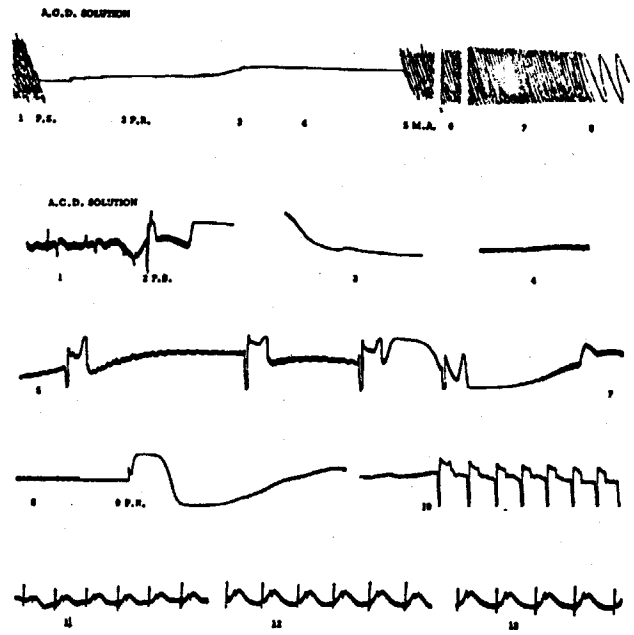

FIG. 5. Tracing of mechanical activity on upper line: *1*, control amplitude of contraction; *P.S.*, perfusion stopped 6 cubic centimeters of A.C.D. solution, 1:2 dilution of stock solution, injected followed by arrest; *2 P.R.*, perfusion restarted after 30 minute arrest; *3*, 35 seconds; *4*, 50 seconds after reperfusion; *5 M.A.*, onset of mechanical activity 1 minute 15 seconds after perfusion restarted; *6*, 1 minute 25 seconds; *7*, 2 minutes 15 seconds after reperfusion with return to maximum amplitude; *8*, 2 minutes 30 seconds after perfusion restarted with tracing at a higher speed of drum rotation to show the shape of the contraction wave. Electrocardiographic tracings: *1*, control; *2 P.S.*, 6 cubic centimeters of A.C.D. solution, 1:2 dilution of stock solution, injection begun; *3*, *4*, during injection; *5*, injection completed; *7*, arrest 7½ seconds after injection; *8*, tracing of arrest; *9 P.R.*, perfusion restarted after 10 minute arrest; *10*, onset of electrical activity 36 seconds after start of reperfusion; *11*, 4 minutes 15 seconds; *12*, 6 minutes; *13*, 8 minutes after perfusion restarted.

electrical activity persisted for 4 seconds after mechanical activity had ceased. Initial electrocardiographic recovery occurred on the average in 20 seconds with return to a regular rhythm in 1 minute and 40 seconds (Fig. 4).

A.C.D. (sodium citrate, citric acid, dextrose) solution. In 15 determinations using 5 cubic centimeters of a 1:2 dilution of this stock solution, mechanical arrest was immediate in 11 instances, took 1.5 seconds in 1 and 0.5 second in 3 others for an average time to arrest of 0.2 second. Initial recovery occurred on the average of 1 minute 31 seconds while maximum recovery required an average of 3 minutes 16 seconds. The initial heart rate averaged 183 beats per minute with an average amplitude of contraction of 2.6 centimeters. At the time of maximum recovery the rate averaged 196 beats per minute. The amplitude returned to an average of 2.26 centimeters, 87.1 per cent of the

initial value (Fig. 5). The periods of arrest varied from 5 to 63 minutes for an average of 26 minutes.

Electrocardiographic determinations done on 4 hearts arrested for an average of 16 minutes each revealed that electrical activity persisted for an average of 10 seconds after mechanical activity had ceased. Initial electrocardiographic recovery occurred on the average in 35 seconds with return to a regular rhythm in 2 minutes 30 seconds (Fig. 5).

Anoxia. In 10 determinations the amplitude of contraction and the heart rate gradually decreased after the flow of perfusion fluid was stopped. Mechanical arrest occurred from 3 minutes 34 seconds to 10 minutes after the clamp was applied for an average time to arrest of 5 minutes 8 seconds. Initial recovery occurred in 47 seconds on the average while maximum recovery required 5 minutes 51 seconds. The initial heart rate averaged 192 beats per minute with an average amplitude of contraction of 2.0 centimeters. At the time of maximum recovery the average rate was 205 beats per minute. The average amplitude of contraction returned to 1.65 centimeters, 82.4 per cent of the initial value (Fig. 6). The periods of arrest varied from 10 to 60 minutes for an average of 22 minutes 1 second. In 1 instance (determination 11) a heart did not recover following 70 minutes of arrest. This preparation had undergone a 60 minute period of potassium citrate arrest 30 minutes earlier.

Electrocardiographic determinations done on 4 hearts arrested for an average of 37 minutes and 30 seconds each revealed that electrical activity persisted long after mechanical activity had ceased. In 1 heart with mechanical arrest for 70 minutes there was electrical activity throughout the period of arrest. At the end of this time, the rate was 36 beats per minute and consisted only of a ventricular complex. When perfusion was restarted fibrillation, both ventricular and auricular, occurred. The heart never recovered a normal electrocardiographic pattern nor did mechanical activity recur. All the hearts showed some degree of fibrillation after removal of the clamp and reperfusion. In some cases this was marked. Return to a regular rhythm occurred on the average in 4 minutes (Fig. 6).

Hypothermia. In 4 determinations using a circulating refrigerant to cool the perfusion bath and the heart chamber, arrest occurred at temperatures of 15.0 to 17.0 degrees C., an average of 16.2 degrees C. Initial recovery after reheating occurred with temperature of perfusion bath at 22 to 26 degrees C., an average of 25.5 degrees C. After initial recovery, there was a gradual increase in amplitude of contraction and heart rate which continued until maximum recovery occurred, usually at the original starting temperature of the perfusion bath, 38 degrees C. The initial heart rate averaged 195 beats per minute with an average amplitude of contraction of 1.8 centimeters. At the time of maximum recovery the rate averaged 176 beats per minute. The amplitude of contraction returned to an average of 1.41 centimeters, 78.2 per cent of the initial value (Fig. 7a). The periods of arrest were 10 minutes. Electrocardiographic determinations on 2 hearts arrested for 10 minutes each revealed that electrical activity ceased at the same time that mechanical activity stopped. Initial electrocardiographic recovery after reheating occurred at average bath temperature of 23 degrees C. Regular electrocardiographic pattern began at an average of 30 degrees C. and continued unchanged except for increase in rate until normal (starting) temperatures had been reached (Fig. 7b).

DISCUSSION

Each of the methods or chemicals tested was capable of producing elective cardiac arrest. Potassium citrate and Sealy's mixture caused immediate mechanical arrest, while acetylcholine required 0.1 second and A.C.D. solution 0.2 second. A period of anoxia averaging 5 minutes 8 seconds was necessary for cardioplegia with this technique. Mechanical recovery as measured by the amplitude of

FIG. 6. Tracing of mechanical activity on upper line: *1*, control amplitude of contraction; *P.S.*, perfusion stopped; *2*, 1 minute; *3*, 3 minutes; *4*, 4 minutes; *5*, 5 minutes; *6*, 6 minutes after perfusion stopped; *7*, mechanical arrest; *8 P.R.*, perfusion restarted after 30 minute arrest; *9*, 30 seconds; *10*, 1 minute; *11*, 5 minutes after perfusion restarted with return to maximum amplitude. Electrocardiographic tracings; *1*, control; *P.S.*, perfusion stopped; *2*, mechanical arrest occurred here at 3 minutes 15 seconds after cessation of perfusion; *3*, 9 minutes; *4*, 16 minutes; *5*, 19 minutes after perfusion stopped (15 minutes 45 seconds after mechanical arrest); *6 M.A.*, mechanical activity occurred at this point, 20 seconds after reperfusion; *7*, fibrillation about 1 minute 30 seconds after perfusion restarted; *8*, regular pattern following fibrillation; *9*, 5 minutes; *10*, 9 minutes; *11*, 15 minutes after reperfusion.

contraction, following potassium citrate arrest was 100 per cent. Subsequent to acetylcholine and Sealy's mixture, the perfused hearts recovered to 93 and 90 per cent of initial amplitude. After use of A.C.D. solution recovery was 87 per cent. Anoxia and hypothermia arrests were followed by 82 and 78 per cent return to initial amplitude of contraction.

Electrocardiographic activity persisted for several seconds after mechanical arrest in all instances. Although mechanical arrest occurred in anoxia, electrical arrest did not occur for as long as 70 minutes following cessation of all contractions. It would seem that electrical activity in anoxia continues until true death of the heart. With all methods, initial recovery is associated with some electrocardiographic irregularities. Initial electrical recovery occurred earliest after the use of acetylcholine and latest after Sealy's mixture. Return to normal or control type of electrocardiogram was seen first after potassium citrate arrest and last after A.C.D. solution. The electrocardiogram after anoxic arrest showed marked fibrillation that lasted several minutes before the normal or control electrocardiogram recurred.

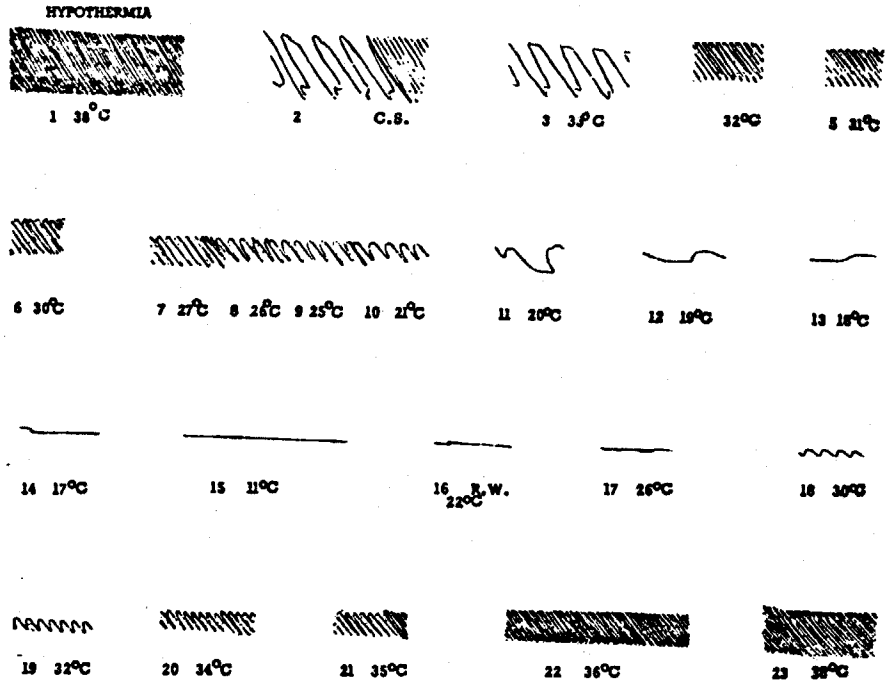

FIG. 7. a, Tracing of mechanical activity: *1*, control amplitude of contraction, temperature 38 degrees C., *2*, *C.S.*, cooling started; *3*, 33 degrees C.; *4*, 32 degrees C.; *5*, 31 degrees C.; *6*, 30 degrees C.; *7*, 27 degrees C.; *8*, 26 degrees C.; *9*, 25 degrees C.; *10*, 21 degrees C.; *11*, 20 degrees C.; *12*, 19 degrees C.; *13*, 18 degrees C.; *14*, 17 degrees C. mechanical activity has ceased; *15*, 11 degrees C. arrest continues; *16*, rewarming has begun after 10 minute arrest at 22 degrees C.; *17*, 26 degrees C. there is some mechanical activity; *18*, 30 degrees C.; *19*, 32 degrees C.; *20*, 34 degrees C.; *21*, 35 degrees C. (tracings from *2* to *21* were taken at drum speed setting of 5 seconds per rotation); *22*, 36 degrees C., with drum rotation setting the same as control tracing; *23*, 38 degrees C. return to maximum amplitude of contraction.

Initial electrocardiographic recovery following hypothermia occurred at a temperature of 23 degrees C. At this time the electrocardiogram showed primarily a poorly defined slow ventricular complex. However, as the temperature rose, the pattern became more normal and was like that of the control when the temperature reached 30 degrees C. Further increase in temperature caused an increase in rate to control ranges without changes in pattern.

The mechanism of arrest by these various agents is not known. It is tempting to explain all of the effects on the basis of accumulation of potassium on the outside of the cell membrane. Such a concept would postulate endogenous and exogenous sources of potassium as the causative agents of arrest. Thus, using potassium citrate or Sealy's mixture, a high extracellular potassium concentration is achieved by the perfusion of the coronary arteries with these potassium rich solutions. In the case of A.C.D. solution, the citrate probably acts on the available extracellular calcium to change the calcium potassium ratio, in essence increasing potassium

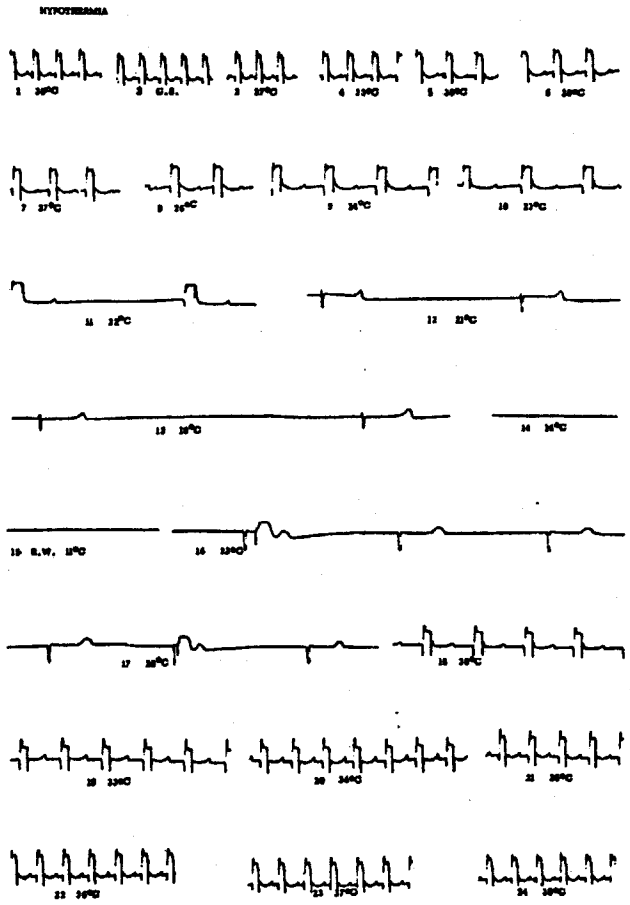

FIG. 7. b, Electrocardiographic tracings: *1*, control at temperature 38 degrees C.; *2 C.S.*, cooling started; *3*, 37 degrees C.; *4*, 33 degrees C., rate is decreasing; *5*, 30 degrees C.; *6*, 28 degrees C.; *7*, 27 degrees C.; *8*, 26 degrees C.; *9*, 24 degrees C.; *10*, 23 degrees C.; *11*, 22 degrees C. marked bradycardia; *12*, 21 degrees C.; *13*, 18 degrees C.; *14*, 16 degrees C., electrical activity has ceased; *15 R.W.*, 11 degrees C., arrest continues, rewarming started; *16*, 23 degrees C., onset of electrical activity; *17*, 28 degrees C. rate increasing; *18*, 30 degrees C. regular rhythm has begun at slow rate; *19*, 33 degrees C.; *20*, 34 degrees C.; *21*, 35 degrees C.; *22*, 36 degrees C.; *23*, 37 degrees C.; *24*, 38 degrees C.

outside the membrane. Hypothermia and anoxia may cause changes in cell membrane permeability or destruction of some cells with liberation of potassium to increase the potassium concentration outside the persisting cell membranes. Acetylcholine may act in a similar fashion, since it is known that this material alters permeability of the cell

membrane to potassium ions, as indicated by Burgen and Terroux.

In attempting to substantiate this elementary concept, potassium determinations were done on the effluate collected during the first minute after starting reperfusion following a period of arrest. These findings compared with control indicate an increased potassium excretion following arrest in all cases. Further work on this point is being done. If the arrests are due to increased extracellular potassium concentrations whether due to an exogenous supply or to an accumulation from an endogenous source, then methods other than those utilizing potassium citrate or Sealy's mixture are achieving arrest as a secondary phenomenon.

On the basis of these studies, potassium citrate appears to be the best cardioplegic agent of the methods tested. It is simple to prepare and store, causes immediate arrest and rapid recovery, and following its use, there is complete return to initial amplitude of contraction and electrocardiographic pattern.

SUMMARY

1. The effects of potassium citrate, Sealy's mixture, acetylcholine, A.C.D. solution, anoxia, and hypothermia on the mechanical and electrical activity of the isolated perfused guinea pig heart have been evaluated and compared.

2. With the exception of anoxia, all of the methods tested cause almost immediate mechanical arrest of the heart followed shortly thereafter by electrical arrest.

3. Average time to mechanical arrest in anoxia was 5 minutes 8 seconds. Electrical arrest did not occur for as long as 70 minutes after anoxia was begun.

4. On reperfusion, initial mechanical activity recurred first after acetylcholine arrest and last after the use of A.C.D. solution. Return to maximum amplitude occurred earliest after potassium citrate and latest following anoxic arrest. Initial electrical activity returned first after acetylcholine and last after Sealy's mixture. There was fibrillation for 1 to 2 minutes when the heart was perfused after anoxia.

5. Using hypothermia, mechanical and electrical arrest was reached at a temperature of 16.2 degrees C. Return to initial mechanical activity occurred at 25.5 degrees C. although electrical activity had begun at 23 degrees C.

6. A possible explanation for the mechanism of arrest with all methods is discussed.

7. On the basis of these studies it is concluded that potassium citrate appears to be the best cardioplegic agent of the methods tested.

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OBSERVATIONS ON THE EFFECT OF PORTACAVAL ANASTOMOSIS UPON THE HEMODYNAMICS OF THE LIVER

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Portacaval anastomosis has had widespread acceptance as the definitive treatment of bleeding esophageal varices. Despite this clinical acceptance only fragmentary data are available concerning the effect of the anastomosis upon hepatic hemodynamics. However, Redeker has recently published circulation studies on 10 cirrhotic patients preceding and following portacaval anastomosis. We have studied 6 patients before and after portacaval surgery. The present article outlines the techniques of study, the findings in these patients, and the integration of these findings with other published material.

MATERIALS AND METHODS

The indications for portacaval anastomosis in these patients were: a previous episode of bleeding esophageal varices in 4, a previous episode of gastrointestinal bleeding and intractable ascites in 1, and intractable ascites in 1. In 5 of the patients cirrhosis developed on an alcoholic, nutritional basis; 1 (T. J.) had postnecrotic cirrhosis.

Prior to operation these patients were studied by hepatic vein catheterization, as previously described (6). On deep inspiration a cardiac catheter was "wedged" in a small hepatic venule. This occlusion of a peripheral venule produces a static column of blood that transmits the pressure in the hepatic sinusoidal bed to the catheter. This intrasinusoidal pressure represents a combined effect of portal vein and hepatic artery pressure. However, this pressure has been shown to be equivalent, in most instances, to portal vein pressure by the methods of Friedman and Reynolds and their associates. In the 2 patients with ascites a correction was made for the intra-abdominal pressure caused by the fluid. The gradient in pressure from the abdominal inferior vena cava to the thoracic inferior vena cava was subtracted from the hepatic wedged pressure. While this may not be entirely correct, in our experience the removal of ascitic fluid generally reduces the wedge pressure to the extent of this abdominal inferior vena cava-thoracic inferior vena cava gradient. Pressures were also taken in the hepatic vein, in the lower inferior vena cava, and right atrium. Following complete diversion of the portal blood flow by end-to-side portacaval anastomosis, the wedged hepatic vein pressure represents the intrasinusoidal pressure developed by the remaining perfusion source, the hepatic artery. Measurements were recorded with a Sanborn electromanometer and direct writing oscillograph; the manometer zero level was taken at an external estimation of the right atrium. Simultaneous blood samples

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Supported in part by a contract between the Office of Naval Research, Department of the Navy, and the State University of New York.

Annales pharmaceutiques françaises 1968, 26, No. 9-10, 593-598

Original Papers

Influence of citrates on renal lithiasis under ethylene glycol

By Ch. Vaille, Ch. Debray, M. Souchard and Cl. Roze (*)

Introduction

The oral administration of ethylene glycol in the male rate produces renal lesions characterized by crystallizations of calcium oxalate monohydrate (20). The females are much less sensitive to this toxin, and the administration of estrogens to male rats protects them against the appearance of renal concretions (15, 17, 18, 19).

We have investigated the effects of the citrate ion on experimental oxalosis of the rat, because on the one hand this acid plays an important part in the calcium metabolism (12) and, on the other, the estrogens cause an increase in the production and oxidation of the blood citrate (16), so that an intermediate effect of this ion in the action of the estrogens on renal oxalosis can be envisaged.

Experimentation

Four lots of ten male Wistar rats (G.N.R.S., Gif) were treated as follows:

1. Controls:

   Food in powder form: "Complete cooked food for rats UAR" (1*)

   Drink: water, ad libitum

2. Ethylene glycol:

   Food in powder form: normal.

   Drink: ethylene glycol at 1% in water.

3. Citrate:

   Food in powder form, prepared as follows:

Complete food	800 g
Citric acid	50 g
Monopotassium citrate (2 H_2O)	75 g
Monosodium citrate (1 H_2O)	75 g

Drink: water

4. Citrate + ethylene glycol.

Citrated diet

Drink: ethylene glycol at 1%.

After one week of observation, during which all animals were given the normal diet, lots 3 and 4 receive for one week the citrated diet alone to avoid a sudden change of diet. Then only ethylene glycol is administered to lots 2 and 4. At the end of three weeks after ethylene glycol, the animals are killed by bleeding the abdominal aorta under ether anesthesia. We determine the urea and the proteins in the serum; we take out and weight the following organs: kidneys, liver, spleen, adrenals, heart, a fragment of which is fixed in 10% formol for histologic examination. During the entire experiment the fluid intake is noted every day, and the weight of the animals measured twice a week. The consumption of citrates is estimated at about 2.5 g per rat and per day.

Results

1. Growth (Table I). - This was an experiment made on animals of rather heavy weight at the start, growing slowly. The results are marked, therefore, by a loss of weight, rather than a slower growth as is the case with younger animals. At the beginning of observation and at the beginning of treatment with citrate, all lots are of homogeneous weight. The citrated diet immediately gives a definite loss of weight, no doubt partially due to the change in calory intake,as this loss of weight then tends to level off. Also the ethylene glycol produces a very significant loss of weight. In the combination ethylene glycol / citrate, the effects of the two factors are not additive, that is, the loss of weight is not greater than with each of the two factors employed alone. The calculation indicates a very significant negative interaction.

2. Fluid intake (Table I).- The quantity of beverage absorbed on the average per rat and per day is substantially the same in the four groups. This, no doubt, has to do with the age of the animals, because in young rats ethylene glycol generally increases the amount of fluid taken.

3. Weight of the organs (Table II).- The spleen, heart, adrenals are not significantly changed, at least when taking into account the body weight of the animals. On the other hand, the kidneys show particularly interesting changes: Citrate alone does not modify their weight, whereas ethylene glycol alone clearly increases it, as is common. With the combination citrate + ethylene glycol, the weight of the kidneys is substantially normal, indicating a significant negative interaction between citrate and ethylene glycol. Also in the liver an effect is noted: Ethylene glycol and citrate both reduce the weight of this organ, more so in the case of the citrate. The combination shows additive effects, not interaction.

Table I. Interaction between ethylene glycol and citrate at forced doses in the rat. Fluid intake and growth.

	Control	E.G.	Citrate	Citrate + E.G.	Difference between groups	E.G. alone	Citrate alone	Interaction
Fluid (ml/rat/day)	23.8	25.1	28.9	29.1	N.S.			
Weight at start of observation (g)	435	422	452	454	N.S.			
Weight at start of citrate (g)	446	434	460	456	N.S.			
Weight at start of E.G. (g)	454	443	409	408	N.S. (0.10)			
Weight on sacrifice (g)	464	380	370	397	0.01	↘ 0.001	↘ 0.001	(-) .001

Table II. Interaction between ethylene glycol and citrate at forced doses in the rat. Weights of the organs, urea, and serum proteins.

	Control	E.G.	Citrate	Citrate + E.G.	Diff. between groups	E.G. alone	Citrate alone	Interaction
Wt. of spleen (1) (mg)	593	561	579	597	N.S.	-	-	-
Wt. of heart (mg)	919	977	974	965	N.S.	-	-	-
Wt. kidneys (cg)	188	284	208	217	0.01	↗ 0.01	N.S.	(-) 0.05

Table II continued

	Control	E.G.	Citrate	Citrate + E.G.	Diff. between groups	E.G. alone	Citrate alone	Inter-action
Wt. liver (cg)	1116	954	872	792	0.001	↓0.05	↓0.001	N.S.
Wt. adrenals (mg)	48.5	48.5	45.0	41.8	N.S.	-	-	-
Urea (2) (cg/l)	44	288	47	47	0.001	0.001	N.S.	(-)0.001
Serum proteins (g/l)	64.8	66.1	65.9	71.3	N.S.	-	-	-

(1) Except in the case of the adrenals, the significance tests are calculated on the weights adjusted on ordinator according to the regression line organ weight/body weight calculated for the controls. The mean weights indicated are the adjusted values.

(2) Significance tests effected after logarithmic transformation.

4. Urea and serum proteins (Table II). The level of blood urea, which is clearly increased by E.G., remains normal only in the case of the combination ethylene glycol + citrate, confirming a strong negative interaction in the kidney and agreeing perfectly with the values of organ weights reported above. The serum proteins are not modified.

5. Histology. The renal lithiasis, the intensity of which is represented by + signs according to a previously described scale (2), is very great in the ten rats treated with ethylene glycol alone: One rat has a lithiasis of ++++, 2 rats of +++, 2 rats of ++, 5 rats of +. It is accompanied by a tubular nephritis in all the intoxicated animals. On the other hand, in the rats treated with citrate combined with ethylene glycol, there is lithiasis only in 4 rats: One rat at +, three others ±; in two of these lithiasic animals the calcium oxalate crystals are accompanied by a slight tubular dilatation and a slight epithelial atrophy, while the other two have no epithelial modifications of the tubes, just as little as do the non-lithiasic rats. The other series of rats have no histological modification of the kidneys. In all series the other organs are substantially normal.

Discussion

The citrates given at strong doses by mouth permit counteracting the nephritis

and the precipitation of calcium oxalate produced by ethylene glycol in the male rat. It is worth mentioning the cause and consequences of this action.

1. Calcium metabolism.- One of the hypotheses envisaged to understand how the calcium salts of the bones are dissolved and pass into the blood is that the citrate forming in the Krebs cycle is the chelating agent in question. The latter would intervene in the equilibrium between the mineral elements of the extracellular liquid and the mineral elements of the bone, with intervention of the parathormone, which would act on the bones through the citric ion to release the calcium (8, 13). One may wonder wheter the increase of the citrate ions in the organism prevents the precipitation of the oxalate in the form of calcium salt in the kidneys as a result of the chelation of the calcium. The calcemia may, in fact, increase in the course of ethylene glycol nephritis (3). Several factors are opposed to this hypothesis. We have shown that the calcemia increases the renal lithiasis from ethylene glycol only when there is a strong intoxication by the dialcohol alone; besides, we have been able to dissociate the lithiasis itself from the hypercalcemia in some experiment series (21).

Moreover, the Harrisons (7) have shown that oral administration of citric acid did not increase the degree of the citremia. Lichtwitz and his associates (15) have summarized the state of the question in these terms: "... the exogenic citric acid passes through the stomach without notable modifications, but it is destroyed later - either during its passage through the intestines, where it is metabolized in the Krebs cycle of the cells of the wall, or in the liver, as the supra-hepatic vein contains less citric acid than the vena portae... It seems, however, that this exogenic citric acid plays an important part in the intestinal absorption of the calcium", in the sense of an augmentation.

Lastly Elliot et al. (6) believe that the role of the citric acid in the normal urinary excretion of the calcium is not very important and practically insignificant in the lithiasis.

As this first hypothesis must be discarded, that of an intervention in the metabolism of the dialcohol remains to be considered.

2. Metalbolism of the ethylene glycol.- Lithiasic intoxication by ethylene

glycol is due essentially to its metabolism leading to the formation of oxalic acid. We have previously put forth the hypothesis that the reduction of oxalosis by the estrogens (19), by phenacitin (21), or by ethanol (4) could be explained by an enzymatic competition where NAD and probably NADP would intervene as coenzyme. Now, while the production of the endogenic citrate depends mostly on NAD, its subsequent oxidation requires NADP (9). It is conceivable, therefore, that the addition per os of strong doses of citrates can cause, either in the intestin or in the liver and probably in both, a strong increase of the coenzyme requirements in the Krebs cycle. Thereby the less metabolized ethylene glycol would be eliminated as such and without producing oxalic acid, hence lithiase.

3. Treatment of acute ethylene-glycol intoxications.- We have suggested, in the case of such an intoxication, to try blocking the enzymes of degradation of the toxin by intercepting them with ethyl alcohol, owing to ethanol perfusions in glucose solution for example (4). Peterson (14) and his associates have increased the LD_{50} of ethylene glycol in the rat by intraperitoneal injection of ethanol.

Wacker et al. (22), following up the results obtained in the treatment of intoxications by methyl alcohol, publish the result of their treatment of ethylene glycol intoxications with ethanol. In France, a demonstration of the fortunate results of this method was made at the Saint Vincent de Paul Hospital in Paris (10, 11). As the doses of ethyl alcohol necessary for treating the ethylene glycol intoxication may be large and an ethyl coma must obviously be avoided, we suggest trying to supplement this detoxication by intravenous ethanol by the administration of strong oral doses of citrates.

4. Treatment of lithiassis by citrates.- Cloarec and Rouffy (1) have treated patients with a strong dose of citric acid and citrates in order to obtain a medicamentous chemolysis of uric lithiasis. They were then able to observe a significant lowering of the concentrations of cholesterol, triglycerides, urea and uric acid in the blood. When Eisenberg, Connord and Howard recommended, in 1955, the use of such a therapy in the treatment of lithiasis, they took as a basis for doing so the hypothesis of a dissolution of the calculi by modification of the urinary pH. If one considers the previous studies on the hyperuricemizing action

of ethanol observed by Delbarre (5), it is permissible to suppose that the activity in the lithiasis of the citrates at strong doses is more likely attributable to a stimulation of the enzymes of the Krebs cycle. In fact, the citrate mixture here used changes but little the urinary pH in the rat, less than sodium bicarbonate at equal doses, and less than Eisenberg's solution, which contains trisodium and tripotassium citrates.

The administration of strong doses of citrate ion in male rats treated with ethylene glycol counteracts the formation of calcium oxalate lithiasis normally obtained under the effect of the dialcohol alone. Also tubular nephritis is prevented, which follos the crystallized deposits in the kidney. This experimentation explains the results obtained with the use of citrates in the therapy of lithiasis. This spectacular action suggests that the use of citrates in strong oral doses may be recommended in the treatment of acute ethylene glycol intoxication.

Bibliography

(Footnotes)

(*) Paper submitted to the Academy of Pharmacy, session of May 8, 1968.

(**) Project sponsored by the I.N.S.E.R.M.

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Translated by Carl Demrick Associates, Inc./JH/db

SÉANCE DU 10 MARS 1968

Recevant le professeur VALETTE, Doyen honoraire de la Faculté de Pharmacie de Paris, professeur de Pharmacodynamie, la Société de Pharmacie de Lille s'est réunie à la Faculté de Médecine et Pharmacie le dimanche 10 mars 1968.

Madame le professeur BÉZANGER eut le plaisir de présenter à l'assemblée ce brillant conférencier qui honore la Pharmacodynamie par sa notoriété, son enseignement et ses recherches. Citons, entre autres orientations de son activité, de remarquables travaux sur l'histamine, le mécanisme d'action des drogues purgatives, l'absorption cutanée de certains médicaments, etc. De réputation mondiale, le professeur VALETTE a été très souvent chargé de missions scientifiques, notamment en Tunisie, Yougoslavie, Moyen-Orient, Extrême-Orient, Canada, Amérique latine. Succédant en 1960 au professeur René FABRE comme Doyen de la Faculté de Pharmacie de Paris, il s'est fait le promoteur très actif de la réforme des Etudes pharmaceutiques.

Le sujet de la conférence était le suivant : *Importance des récepteurs adrénergiques dans le mécanisme d'action de certains médicaments.* — Depuis la découverte des propriétés hypertensives du sang veineux surrénal dans la deuxième moitié du XIXe siècle, de nombreux travaux ont été effectués. Ils aboutirent, vers 1950, à la mise en évidence du rôle primordial de la noradrénaline. Celle-ci, comme toutes les catécholamines, présente des réactions de fluorescence qui peuvent être utilisées pour sa caractérisation au niveau des cellules : la noradrénaline est [illegible] dans les vésicules synaptiques, visibles au microscope électronique.

[illegible] de la noradrénaline est primordial dans le fonctionnement des fibres sympathiques chez les animaux supérieurs. Par contre, chez les Batraciens, l'adrénaline a autant d'importance que la noradrénaline.

Du point de vue biosynthèse, la noradrénaline provient de la transformation de la tyrosine sous l'influence de certains enzymes. Quant à la dégradation de l'adrénaline et de la noradrénaline, elle s'effectue en présence de monoamineoxydase (M. A. O.) et de catéchol-O-méthyltransférase (C. O. M. T.). Cette dernière agit surtout sur l'adrénaline et la noradrénaline circulantes.

Les surrénales libèrent l'adrénaline, les terminaisons nerveuses la noradrénaline.

Il existe des substances entravant la formation du médiateur : ce sont les anti-adrénergiques (métyrosine, guanéthidine, qui empêchent la propagation de l'influx nerveux). A noter que la reserpine, dont on connait l'utilisation en thérapeutique, provoque une déplétion très prolongée des tissus en noradrénaline : elle empêche le stockage du médiateur.

En ce qui concerne la noradrénaline, on admet la présence de récepteurs α et β donnant des effets α et β inhibés par des substances différentes. Ce phénomène explique l'activité de drogues telles que l'Ergot de seigle, à la fois sympatholytique et vasoconstricteur.

M. le professeur A. LESPAGNOL félicita M. le professeur VALETTE pour sa très belle conférence, suivie avec le plus grand intérêt par toute l'assemblée, puis il prit la parole pour présenter un *Ensemble de recherches dans la série des antispasmodiques*, réalisé avec ses collaborateurs en faisant l'objet de plusieurs thèses de Doctorat dans son service.

L'ordre du jour étant épuisé, la séance fut levée vers midi.

M. PINKAS.

Printed in France. *Le Directeur de la Publication* : G. MASSON.
Dépôt légal. — 1968. — 4e trimestre. — N° d'ordre 4365. — MASSON et Cie, éditeurs, Paris.
Imprimé par l'Ancienne Imprimerie de la Cour d'Appel, 1, rue Cassette à Paris (France).

Annales pharmaceutiques françaises
1968, 26, n° 9-10, pp. 593-598

MÉMOIRES ORIGINAUX

Influence des citrates sur la lithiase rénale à l'éthylène-glycol.

par

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INTRODUCTION

L'administration orale d'éthylène-glycol chez le Rat mâle produit des lésions rénales caractérisées par des cristallisations d'oxalate de calcium monohydraté [20]. Les femelles sont beaucoup moins sensibles à ce toxique, et l'administration d'œstrogènes aux rats mâles les protège contre l'apparition des concrétions rénales [15, 17, 18, 19].

Nous avons recherché les effets de l'ion citrate sur l'oxalose expérimentale du Rat, car d'une part cet acide joue un rôle important dans le métabolisme du calcium [12] et, d'autre part, les œstrogènes provoquent à la fois une augmentation de la production et de l'oxydation du citrate sanguin [16], permettant ainsi d'envisager un effet intermédiaire de cet ion dans l'action des œstrogènes sur l'oxalose rénale.

EXPÉRIMENTATION

Quatre lots de dix rats mâles de souche Wistar (C. N. R. S., Gif) ont été traités de la manière suivante :

1° TÉMOINS :

Nourriture en poudre : « aliment cuit complet pour rats UAR » (1).

Boisson : eau *ad libitum*.

(*) Mémoire présenté à l'Académie de Pharmacie, séance du 8 mai 1968.
(**) Travail réalisé avec l'aide de l'I. N. S. E. R. M.
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2° ÉTHYLÈNE-GLYCOL :

Nourriture en poudre : normale.

Boisson : éthylène-glycol à 1 % dans l'eau.

3° CITRATE :

Nourriture en poudre, préparée comme suit :

Aliment complet	800 g
Acide citrique	50 g
Citrate monopotassique à 2 H_2O	75 g
Citrate monosodique à 1 H_2O	75 g

Boisson : eau.

4° CITRATE — ÉTHYLÈNE-GLYCOL :

Régime citraté.

Boisson : éthylène-glycol à 1%.

Après une semaine d'observation, pendant laquelle tous les animaux sont soumis au régime normal, les lots 3 et 4 reçoivent pendant une semaine le régime citraté seul pour éviter un changement brutal de régime. A ce moment seulement l'éthylène-glycol est administré aux lots 2 et 4. Au bout de trois semaines après éthylène-glycol, les animaux sont tués par saignée à l'aorte abdominale sous anesthésie à l'éther. Nous dosons l'urée et les protides dans le sérum ; nous prélevons et pesons les organes suivants : reins, foie, rate, surrénales, cœur dont un fragment est fixé au formol 10 % pour l'examen histologique. Pendant toute la durée de l'expérience, la consommation de boisson est notée tous les jours, et le poids des animaux mesuré deux fois par semaine. La consommation de citrates est estimée à 2.5 g environ par rat et par jour.

RÉSULTATS

1° *Croissance* (tableau I). — Il s'agit d'une expérience conduite sur des animaux de poids assez fort au départ, dont la croissance est lente. Les résultats se marqueront donc par un amaigrissement, au lieu d'un ralentissement de croissance, comme c'est le cas

TABLEAU I. — *Interaction entre l'éthylène-glycol et le citrate à doses forcées chez le Rat. Boisson et croissance.*

	TÉMOINS	E.G.	CITRATE	CITRATE + E.G.	DIFFÉRENCE ENTRE GROUPES	E.G. SEUL	CITRATE SEUL	INTERACTION
Boisson (ml rat/jour)	23,8	25,1	28,9	29,1	N.S.			
Poids début observation (g)	435	422	452	454	N.S.			
Poids début citrate (g)	446	434	460	456	N.S.			
Poids début E. G. (g)	454	443	409	408	N.S. (0.10)			
Poids sacrifice (g)	464	380	370	397	0,01	↘ 0,001	↘ 0,001	(—) 0,001

pour les animaux plus jeunes. En début d'observation et en début de traitement au citrate, tous les lots sont de poids homogène. Le régime citraté donne immédiatement un amaigrissement net, sans doute partiellement dû au changement de l'équilibre calorique des apports, car cet amaigrissement tend ensuite à s'estomper. L'éthylène-glycol produit lui aussi une perte de poids très significative. Dans l'association éthylène-glycol citrate, les effets des deux facteurs ne sont pas additifs, c'est-à-dire que la perte de poids n'est pas plus forte qu'avec chacun des deux facteurs employés seuls. Le calcul fait apparaître une interaction négative très significative.

2° *Boisson* (tableau I). — La quantité de boisson absorbée en moyenne par Rat et par jour est sensiblement la même dans les quatre groupes. Cela tient sans doute à l'âge des animaux, car chez les jeunes rats, l'éthylène-glycol augmente généralement l'absorption de boisson.

3° *Poids des organes* (tableau II). — La rate, le cœur, les surrénales ne sont pas modifiés de manière significative, au moins lorsque l'on tient compte du poids corpo-

TABLEAU II. — *Interaction entre l'éthylène-glycol et le citrate à doses forcées chez le Rat. Poids des organes, urée et protides sériques.*

	TÉMOINS	E.G.	CITRATE	CITRATE + E.G.	DIFFÉRENCE ENTRE GROUPES	E.G. SEUL	CITRATE SEUL	INTERACTION
Poids rate (1) [mg]	593	561	579	597	N.S.	—	—	—
Poids cœur (mg)	919	977	974	965	N.S.	—	—	—
Poids reins (cg)	188	284	208	217	0,01	↗ 0,01	N.S.	(—) 0,05
Poids foie (cg)	1116	954	872	792	0,001	↘ 0,05	↘ 0,001	N.S.
Poids surrénales (mg)	48,5	48,5	45,0	41,8	N.S.	—	—	—
Urée (2) [cg/l]	44	288	47	47	0,001	0,001	N.S.	(—) 0,001
Protides sérum (g/l)	64,8	66,1	65,9	71,3	N.S.	—	—	—

(1) Sauf dans le cas des surrénales, les tests de signification sont calculés sur les poids ajustés sur ordinateur d'après la droite de régression poids d'organe poids du corps calculée pour les témoins. Les poids moyens indiqués sont les valeurs ajustées.

(2) Tests de signification effectués après transformation logarithmique.

rel des animaux. Par contre, les reins présentent des variations particulièrement intéressantes : le citrate seul ne modifie pas leur poids, tandis que l'éthylène-glycol seul l'augmente nettement, comme il est habituel. Avec l'association citrate et éthylène-glycol, le poids des reins est sensiblement normal, ce qui indique une interaction négative significative entre citrate et éthylène-glycol. Au niveau du foie, s'observe également une action : éthylène-glycol et citrate diminuent tous deux le poids de cet organe, davantage dans le cas du citrate. La combinaison montre des effets additifs et pas d'interaction.

4° *Urée et protides du sérum* (tableau II). — Le taux de l'urée sanguine, nettement augmenté par l'E. G., seul reste normal dans le cas de l'association éthylène-gly-

col citrate, ce qui confirme au niveau du rein une forte interaction négative et s'accorde parfaitement avec les valeurs de poids des organes rapportées plus haut. Les protides sériques ne sont pas modifiés.

5° *Histologie.* — La lithiase rénale dont l'intensité est figurée par des +, selon une échelle décrite précédemment [2], est très importante chez les dix rats traités à l'éthylène-glycol seul : 1 rat a une lithiase à + + + +, 2 rats à + + +, 2 rats à + +, 5 rats à +. Elle est accompagnée d'une néphrite tubulaire chez tous les animaux intoxiqués. Par contre, chez les rats traités par du citrate associé à l'éthylène-glycol il y a une lithiase seulement chez 4 rats : un rat à +, trois autres ± ; chez deux de ces animaux lithiasiques les cristaux d'oxalate de calcium sont accompagnés d'une légère dilatation tubulaire et d'une légère atrophie épithéliale ; tandis que les deux autres, pas plus que les rats non lithiasiques, n'ont de modifications épithéliales des tubes. Les autres séries de rats n'ont pas de modification histologique rénale. Les autres organes sont dans toutes les séries sensiblement normaux.

Commentaires

Les citrates administrés à doses fortes par voie buccale permettent de s'opposer à la néphrite et à la précipitation d'oxalate de calcium produites par l'éthylène-glycol chez le Rat mâle. La cause et les conséquences de cette action méritent d'être évoquées.

1° *Métabolisme du calcium.* — Une des hypothèses envisagées pour comprendre comment les sels calciques des os sont dissous et passent dans le sang est que le citrate se formant dans le cycle de Krebs est l'agent chélateur en cause. Celui-ci interviendrait dans l'équilibre entre les éléments minéraux du liquide extracellulaire et les éléments minéraux de l'os, avec intervention de la parathormone qui agirait sur les os par l'intermédiaire de l'ion citrique pour libérer le calcium [8, 13]. Nous pouvons nous demander si l'augmentation des ions citrates dans l'organisme n'empêche pas la précipitation de l'oxalate sous forme de sel de calcium dans les reins par suite de la chélation du calcium. La calcémie peut, en effet, s'élever au cours de la néphrite à l'éthylène-glycol [3]. Plusieurs éléments s'opposent à cette hypothèse. Nous avons montré que la calcémie n'augmente dans la lithiase rénale à l'éthylène-glycol que lorsqu'il y a une forte intoxication par le diacool seul ; en outre, nous avons pu dissocier la lithiase elle-même de l'hypercalcémie dans certaines séries d'expériences [21].

De plus, les Harrison [7] ont démontré que l'administration *per os* d'acide citrique n'augmentait pas le taux de la citrémie. Lichtwitz et ses collaborateurs [15] ont résumé l'état de la question dans les termes suivants : « ... l'acide citrique exogène traverse l'estomac sans modifications notables, mais il est détruit ultérieurement : soit dans sa traversée intestinale où il est métabolisé dans le cycle de Krebs des cellules de la paroi, soit dans le foie, car la veine sus-hépatique contient moins d'acide citrique que la veine porte... Il semble par contre, que cet acide citrique exogène joue un rôle important dans l'absorption intestinale du calcium », dans le sens d'une augmentation.

Enfin Elliot et ses collaborateurs [6] pensent que le rôle de l'acide citrique dans l'excrétion urinaire normale du calcium est peu important et pratiquement insignifiant dans la lithiase.

Cette première hypothèse devant être écartée, il reste à envisager celle d'une intervention dans le métabolisme du diacool.

2° *Métabolisme de l'éthylène-glycol.* — L'intoxication lithiasique par l'éthylène-gycol est due essentiellement à son métabolisme conduisant à la formation d'acide oxalique. Nous avons émis précédemment l'hypothèse que la diminution de l'oxalose par les œstrogènes [19], par la phénacétine [21], par l'éthanol [4] pouvait s'expliquer par une compétition enzymatique où interviendraient comme coenzyme NAD et probablement NADP. Or, si la production du citrate endogène dépend surtout de NAD, son oxydation ultérieure nécessite NADP [9]. Nous pouvons donc imaginer que l'adjonction *per os* de doses fortes de citrates puisse, soit au niveau de l'intestin, soit au niveau du foie et probablement des deux, provoquer une forte augmentation des besoins en coenzymes au niveau du cycle de Krebs. De ce fait, l'éthylène-glycol, moins métabolisé, s'éliminerait tel quel et sans produire d'acide oxalique, donc de lithiase.

3° *Traitement des intoxications aiguës à l'éthylène-glycol.* — Nous avons suggéré, en cas d'une telle intoxication, d'essayer de bloquer les enzymes de dégradation du toxique en les captant par l'alcool éthylique, grâce à des perfusions d'éthanol dans du soluté glucosé par exemple [4]. Peterson [14] et ses collaborateurs ont augmenté la dose léthale 50 de l'éthylène-glycol chez le Rat par injection intrapéritonéale d'éthanol.

Wacker et ses collaborateurs [22], s'inspirant des résultats obtenus dans le traitement des intoxications par l'alcool méthylique, publient le résultat de leur traitement par l'éthanol d'intoxications à l'éthylène-glycol. En France, une démonstration des heureux résutats de cette méthode a été faite à l'hôpital Saint-Vincent-de-Paul, à Paris [10, 11]. Etant donné que les doses d'alcool éthylique nécessaires au traitement de l'intoxication par l'éthylène-glycol peuvent être importantes et qu'il faut évidemment éviter de provoquer un coma éthylique, nous suggérons d'essayer de compléter cette désintoxication par l'éthanol intraveineux par l'administration de citrates à fortes doses par voie buccale.

4° *Traitement des lithiases par les citrates.* — Cloarec et Rouffy [1] ont traité des malades avec une forte dose d'acide citrique et de citrates dans le but d'obtenir une chémolyse médicamenteuse de lithiases uriques. Ils ont pu alors constater un abaissement significatif des taux sanguins du cholestérol, des triglycérides, de l'urée et de l'acide urique. Lorsque Eisenberg, Conord et Howard ont préconisé en 1955, l'emploi d'une thérapeutique de ce genre dans le traitement de la lithiase, ils se basaient pour ce faire sur l'hypothèse d'une dissolution des calculs par modification du *p*H urinaire. Si l'on rapproche les travaux précédents de l'action hyperuricémiante de l'éthanol observée par Delbarre [5], il est permis de supposer que l'activité dans la lithiase des citrates à doses fortes est plus vraisem-

blablement attribuable à une stimulation des enzymes du cycle de KREBS. En effet, le mélange de citrates utilisé ici modifie peu le *p*H urinaire chez le Rat, moins que le bicarbonate de sodium à doses égales, et moins que la solution d'EISENBERG, qui contient des citrates trisodique et tripotassique.

L'administration de doses fortes d'ion citrate à des rats mâles traités par de l'éthylène-glycol s'oppose à la création de la lithiase à l'oxalate de calcium habituellement obtenue sous l'effet du seul diglycol. De même est empêchée la néphrite tubulaire qui suit les dépôts cristallisés dans le rein. Cette expérimentation explique les résultats obtenus par l'emploi des citrates dans la thérapeutique des lithiases. Enfin, cette action spectaculaire permet de penser que l'emploi de citrates à doses fortes *per os* peut être préconisé dans le traitement de l'intoxication aiguë à l'éthylène-glycol.

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Annales pharmaceutiques françaises
1968, **28**, n° 9-10, pp. 599-602

Analyse des pommades.

VI. — *Etude de la composition du blanc de baleine par spectrométrie de masse,*

par

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RÉSUMÉ. — *Une analyse très fine d'un échantillon de blanc de baleine a été réalisée par chromatographie d'adsorption sur silice, suivie d'un examen des groupes chimiques ainsi séparés par spectrométrie de masse. Cette méthode a permis l'identification d'un nombre important de constituants non encore signalés jusqu'ici.*

Dans une récente publication [2] nous avions proposé une méthode d'analyse des cires en prenant comme exemple le blanc de baleine. Ce dernier avait été séparé en plusieurs fractions par chromatographie d'adsorption sur silice. La composition de chacune des fractions avait été étudiée par la chromatographie sur couches minces, la spectroscopie infra-rouge, la résonance magnétique nucléaire et la chromatographie en phase gazeuse. En dehors de traces d'hydrocarbures, trois groupes de constituants principaux avaient alors été isolés : des esters d'acides et d'alcools gras (fraction A), des triglycérides (fraction B) et un mélange d'acides et d'alcools libres accompagnés d'hydroxyesters (fraction C). Dans chacun de ces groupes, la chromatographie en phase gazeuse avait permis d'identifier les constituants indiqués dans le tableau I.

Le but du présent travail est d'approfondir l'étude de la composition du blanc de baleine en ayant recours à la spectrométrie de masse.

La spectrométrie de masse a été effectuée en utilisant un appareil Atlas CH_4 avec une source d'ions pour solides TO_4. Les spectres ont été enregistrés en utilisant une tension d'ionisation non seulement de 70 eV mais aussi de 11 eV de façon à

(*) Manuscrit reçu le 13 novembre 1967.

Scot. med. J., 1962, 7: 306

THE EFFECTS OF POTASSIUM CITRATE ARREST ON THE DOG'S HEART

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DURING open-heart surgery, Melrose and his associates (1955) induced cardiac arrest with potassium citrate. This method which has been widely adopted in clinical practice involves the injection of an aqueous solution of potassium citrate solution into the aorta proximal to a clamp applied distal to the coronary ostia.

Thereafter several workers have described a distinct type of necrosis in the hearts of many of those subjected to potassium citrate arrest: the lesions consisted of multiple, sharply circumscribed, microscopic areas of necrosis situated usually in the central portion of the ventricular myocardium. McFarland *et al.* (1930) examined the hearts of thirty patients who died after intracardiac operation with cardiopulmonary bypass. Nineteen patients had cardiac arrest induced electively by the Melrose technique, using 20 to 90 ml. of 2.5 per cent potassium citrate, the aorta being clamped for periods of 6 to 67 minutes. The more severe lesions followed a mean time of arrest of 23 minutes (12-67 min.), and the less severe lesions a mean time of arrest of 18 minutes (6-30 min.). They claimed that even in patients dying 2 hours or less after cardiac arrest there was already evidence of necrosis.

The purpose of the investigation was to study the effects of potassium citrate on the myocardium of the dog, under experimental conditions, since factors other than potassium citrate might have caused the myocardial necrosis reported.

METHODS

Total cardiopulmonary bypass using the Melrose Pump Oxygenator was instituted in nineteen mongrel dogs, the weights of which ranged from 12.9 to 36 kg. The temperature of the body was kept as near normal as possible. Blood pressures in the superior and inferior vena cava and a systemic artery were recorded to assist the control of the bypass. A continuous electrocardiographic record was also made. The blood flow into the cannulated femoral artery was at the rate of 80 ml. per kg. body weight per min. The blood volume of the dogs was kept constant by the maintenance of normal venous pressure, and by accurate measurement and replacement of blood loss. The body temperature did not fall more than 4°C. during bypass.

Arterial blood pH (Wynn & Ludbrook, 1957) and the total plasma CO_2 content (Van Slyke & Neill, 1924) were measured intermittently. From these values, pCO_2 and buffer base were calculated using the Singer-Hastings nomogram (Singer & Hastings, 1948). In arterial and venous blood samples oxygen saturation was determined by a Brinkman Haemoreflector.

Immediately following the cessation of the bypass, systemic arterial hypotension usually occurred, but in all cases myocardial contractions improved steadily and systemic arterial pressure returned to pre-bypass levels in 2 to 3 minutes. The dogs were divided into three groups: in Group I the effects of potassium citrate and associated aortic occlusion were studied; in Group II (as a control) the effects of bypass with aortic occlusions for 0 to 5 minutes were noted; in Group III the effects of a long period of bypass with long periods of aortic occlusion were examined.

Group I comprised six mongrel dogs (wt 15-28.5 kg.) in which, after initial taping, the aorta was clamped and cardiac arrest induced by the Melrose technique, using 2.5 per cent potassium citrate solution. This potassium citrate solution was freshly prepared by diluting 2 ml. of 25 per cent potassium citrate with 18 ml. of homologous blood. In all dogs asystole followed within 2, and usually within 1 minute of the injection of the potassium citrate. Cardic arrest lasted for 20 to 2[illegible] minutes (Table 1). Right ventriculotomy with incision and closure of the ventricular septum

Table I. Animals subjected to potassium citrate arrest and aortic occlusion for 20 minutes (*Group I*).

Dog no.	Weight (kg.)	Duration of bypass (min.)	Duration of vent-riculotomy (min.)	Duration of aortic clamping (min.)	Volume of 2.5% potassium citrate (ml.)	Duration of life after bypass	Myocardial histology	Metabolic acidosis
1	17.0	39	28	20	10	12 hr.	Normal	Present
2	28.4	35	20	20	20	24 hr.	Normal	Present
3	17.0	51	15	23	20	3 days	Normal	Absent
4	16.0	38	22	23	20	10 days	Normal	Present
5	15.0	33	27	20	20	15 days	Normal	Absent
6	17.3	46	30	20	20	20 days	Normal	Present

Table II. Animals subjected to 5 minutes of aortic occlusion (*Group II*).

Dog no.	Weight (kg.)	Duration of bypass (min.)	Duration of ventri-culotomy (min.)	Duration of aortic clamping (min.)	Duration of life after bypass	Myocardial histology	Metabolic acidosis
7	14	34	15	5	6 hr.	Normal	Absent
8	18	28	14	5	10 hr.	Normal	Absent
9	14	20	10	5	4 wks.	Normal	Absent
10	14	28	15	5	10 wks.	Normal	Absent
11	12.9	52	49	0	6 hr.	Normal	Present

Table III. Animals subjected to intermittent aortic occlusion for 15 to 35 minutes (*Group III*).

Dog no.	Weight (kg.)	Duration of bypass (min.)	Duration of ventri-culotomy (min.)	Duration of aortic clamping (min.)	Duration of life after bypass	Myocardial histology	Metabolic acidosis
12	13	38	21	15	6 hr.	Normal (mural thrombus present)	Absent
13	16.7	45	25	15	12 hr.	Normal	Present
14	19.0	36	25	15	2 wks.	Normal (mural thrombus present)	Present
15	20.2	38	21	20	2 hr.	Normal	Absent
16	26.5	52	22	20	12 hr.	Normal	Present
17	12.7	48	40	30	6 hr.	Normal	Absent
18	36	52	30	35	6 hr.	A few necrotic muscle fibres	Present
19	17	50	40	30	14 hr.	Normal	Absent

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was then carried out. Bypass was maintained for an average duration of 40 minutes (range 33-51 min.). On release of the aortic clamp, ventricular fibrillation occurred in five of the six dogs but defibrillation was easily accomplished. In the remaining dog the aortic release was followed by the return of normal sinus rhythm.

These dogs were killed at varying intervals after the operation, and the hearts were examined histologically for evidence of myocardial damage. From all parts of the heart including portions of the wall of each chamber eight to twelve blocks were taken; at least three blocks were taken from the left ventricle. The tissues were fixed in 10 per cent formol saline and stained with haematoxylin and eosin.

Group II consisted of five mongrel dogs (wt. 12.9-18 kg.). The aorta was clamped distal to the coronary ostia for 5 minutes in four dogs. In the remaining dog the aorta was not clamped. The duration of bypass was shorter than in Group I and the ventricular septum was not opened. These dogs' hearts were examined histologically after death or sacrifice.

Group III consisted of eight mongrel dogs (wt. 13-36 kg.). The aorta was clamped intermittently distal to the coronary ostia for periods of 15, 20 and 30 minutes. The aortic clamp was released for 1 minute in every 5 minutes. The duration of bypass and of right ventriculotomy was longer than in the other two groups. Ventricular septal incision and suture was carried out in only three of these dogs. All these dogs' hearts were examined histologically after death or sacrifice.

RESULTS

Group I. (Table I.) Histological examination of the hearts of dogs perfused with potassium citrate showed no evidence of myocardial damage except at the ventriculotomy and septal incision sites, where organizing areas of necrosis were found. No lesion resembling those described by McFarland *et al.* (1960) was seen.

Group II. (Table II.) There was no abnormality of the myocardium in these hearts on histological examination except at the sites of ventriculotomy.

Group III. (Table III.) In the heart of dog no. 12 there was an organizing mural thrombus (3 × 2 × 1.5 cm.) on the endocardial surface of the ventriculotomy wound in the right ventricle. In the heart of dog no. 14 an adherent mural thrombus measuring 2.5 × 1.5 cm. was found at the ventriculotomy site, and there was more than the expected amount of necrotic muscle at the operation sites. In dog no. 18, microscopic examination of the heart showed muscle necrosis at the operation site and subendocardial haemorrhage with some oedema in the region of the left ventricle. A few subendocardial muscle fibres appeared degenerate with swelling and vacuolation of cytoplasm and a few were necrotic. None of the other hearts showed any unexpected changes and there was no evidence of myocardial necrosis.

Metabolic acidosis occurred in nine out of nineteen dogs. There was no correlation between the degree of metabolic acidosis and the minimal microscopical myocardial changes found.

DISCUSSION

Helmsworth *et al.* (1959) found evidence of myocardial necrosis in four dogs subjected to elective cardiac arrest for 30 minutes by the method of Melrose *et al.* (1955), but in two dogs subjected to the same duration of arrest by coronary occlusion alone they did not find these lesions. Bjork *et al.* (1961) found similar lesions in two dogs after elective cardiac arrest with potassium citrate for 30 to 35 minutes.

On the other hand, Redo (1959) compared various cardioplegic agents using the Langendorff preparation in the guinea pig heart, and found that potassium citrate was a better agent than hypothermia, anoxia or acid citrate dextrose. Under these conditons potassium citrate produced immediate cardiac arrest and recovery of heart action was more rapid than with the other agents. This supported the work of Melrose (1955), who showed that cardioplegia for 5 minutes by coronary occlusion alone was followed by a slower recovery of heart action than was cardioplegia for 15 minutes using coronary occlusion and potassium citrate.

Our studies indicated that periods of elec-

tive cardiac arrest with potassium citrate of up to 20 minutes duration did not cause microscopic myocardial damage or necrosis. The dosage used in our series did not exceed 20 ml. of 2.5 per cent of potassium citrate and the weight of the heart in the lightest dog (wt. 13 kg.) was 150 g.

It is of interest to note that five out of the 6 dogs in the group undergoing elective potassium arrest for 20 minutes were long-term survivors, whereas a much higher mortality occurred with intermittent aortic clamping. This does not accord with the findings of Kaplan and his associates (1958) who compared the survival rates in dogs perfused with and without elective potassium citrate arrest. Mortality is, however, an unreliable index, since the causes of post-bypass death are often multiple, and in only two of our dogs was death attributable to a cardiac cause.

In clinical practice myocardial necrosis has been reported by McFarland *et al.* (1960) in the hearts of patients dying after elective potassium citrate arrest, but the dosage of potassium citrate, the duration of aortic clamping and the severity of the accompanying procedures were very variable; in such circumstances potassium citrate cannot, with certainty, be identified as the causative agent. McFarland *et al.* suggested that 25 per cent potassium citrate in ampoules had deteriorated but analysis of the potassium citrate used in these experiments showed no deterioration.

There are several explanations of the absence of myocardial changes in the dogs' hearts in our studies in contrast to other published reports. It may be that the dilution effect obtained by injecting the potassium citrate into the left ventricle instead of into the proximal aorta prevented the myocardial damage, but its concentration was still sufficient to provide, on each occasion, immediate arrest. It is also possible that the duration of cardiac arrest may be important since none of our dogs were subjected to more than 23 minutes of such arrest, whereas in the series of Helmsworth *et al.* (1959), and of Bjork *et al.* (1961), the duration of cardiac arrest was 30 minutes or more. Weslowski *et al.* (1952) moreover state that the accepted upper limit of ischaemia of the dog's heart at normal temperature is 20 minutes.

The use of potassium citrate has practical disadvantages: first the onset of heart block cannot be identified during reparative surgery; secondly, its usefulness may be limited to 20 minutes, and, thirdly, there is some evidence that ventricular function is more severely reduced after elective potassium citrate arrest than after intermittent clamping of the aorta (Cooper *et al.*, 1959; Waldhausen *et al.*, 1960).

SUMMARY

Evidence is presented that elective cardiac arrest for 20 minutes by intracardiac injection of 2.5 per cent potassium citrate does not cause microscopic myocardial lesions in the dog.

In contrast, other workers have shown that elective cardiac arrest for 30 minutes may cause myocardial necrosis.

The practical disadvantages of potassium citrate arrest are discussed.

ACKNOWLEDGEMENTS. We are grateful to the Department of Anaesthetics, The Royal Infirmary, Edinburgh for their invaluable assistance in these studies, also to Mr J. Walker and Miss Jean Barclay of the Cardio-Pulmonary Bypass Unit for their technical assistance.

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PNEUMATOSIS CYSTOIDES INTESTINALIS

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PNEUMATOSIS cystoides intestinalis is an uncommon condition of the alimentary tract characterized by the presence of gas-containing cysts in the wall of the bowel. The disorder has long been recognized in hogs; the first classical description in man has been ascribed to Bang (1876) but it had been described before this by Du Vernoi in 1738 and by Cloquet in 1820. It is relatively commoner in the small bowel than in the colon, and Koss (1952) in a comprehensive review of the literature, found only thirteen cases of colonic involvement out of a total of 255. With the increasing awareness of the condition and with improved diagnosis, several other cases involving the colon have since been reported.

The cysts may be present in any layer of the intestinal wall but are usually subserosal or submucosal. They may, therefore, appear on the external surface of the gut or may project into the lumen. They vary in size from 0.1 to 1.0 cm., being smallest in the jejunum and ileum and largest in the distal colon. In children they are more likely to be submucosal, whereas in adults the majority of reported cases have been subserosal. Similar cysts have been reported in the mesentery, omentum, abdominal wall, bladder wall and vagina. According to Koss (1952) the cysts are at least partly lined by a layer of endothelial cells with occasional multinucleated giant cells. The giant cells tend to disappear as fibrosis develops and the cyst lining atrophies. Inflammatory changes occur in the surrounding connective tissue with the production of granulomas and further fibrosis. Eventually there is contraction with scarring, and reduction in size of the cysts as the contained gas is absorbed.

Until recently, the majority of cases have been diagnosed incidentally at laparotomy or at post-mortem examination though a few have been seen on sigmoidoscopy. An increasing number of cases are now being diagnosed by radiological examination, especially when the colon is involved, and in view of the importance of recognizing the condition preoperatively, the following case was considered worthy of report.

Case report. E. S., female, aged 74 years. The patient was first admitted to hospital in 1953 complaining of vomiting and of acute pain in the left lumbar and lower abdominal areas. This acute episode rapidly subsided and all investigations, including barium enema, were normal. She had had enteric fever in 1906, a history of two abortions and had been treated for pernicious anaemia since 1944; the latter diagnosis was subsequently confirmed by gastric analysis and Schilling test.

She was seen again in 1958 complaining of intermittent bouts of blood-tinged diarrhoea, having previously been rather constipated for several years. There had been slight ill-defined abdominal discomfort but no anorexia and no weight loss. Several similar episodes occurred between 1958 and 1961 with occasional attacks of more severe abdominal pain, but these were of short duration and were not accompanied by vomiting. Clinical examination was singularly unhelpful and she continued to maintain normal weight.

Sigmoidoscopy on several occasions showed similar appearances of a slightly dilated, rigid bowel lumen, with healthy mucosa, without evidence of ulceration. At about 20 cm. from the anus, several smooth sessile rounded mucosal swellings about 1 cm. in diameter were noted. Biopsies were taken and showed consistently the same picture. 'The mucous membrane is raised into a small blunt polypoidal structure by a growth of granulation tissue in the submucosa. The granulating region is heavily infiltrated with polymorphs and round cells, mainly the latter, but this feature is overshadowed by a narrow zone of multinucleated giant forms gathered round a sharply-defined space, now empty apart from serous fluid and blood. The mucosa shows marked oedema, corresponding separation of the glands, some infiltration with melanophores and a narrow zone of fat at the surface' (Fig. 1).

Prior to her last admission in 1961, the patient had noticed some progressive but gradual abdominal swelling and examination suggested the presence of ascites. The history of recurrent diarrhoea had raised the possibility of ulcerative colitis with pseudopolyposis and with the onset of possible ascites, it was suspected that she had developed an adenocarcinoma of the colon with peritoneal involvement, but radiological examination and the subsequent good health of the patient proved this to be incorrect.

COMPARATIVE EFFECT OF 15 PER CENT POTASSIUM CHLORIDE AND 30 PER CENT POTASSIUM CITRATE IN RESUSCITATION FROM VENTRICULAR FIBRILLATION FOLLOWING ACUTE MYOCARDIAL INFARCTION

An Experimental Study

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THERE have been relatively few experimental observations on resuscitation of the heart following acute cyocardial infarction.[10, 11, 14, 15, 17] Previous work by one of us (F. W.) has demonstrated the relative effectiveness of 15 per cent potassium chloride in converting ventricular fibrillation to ventricular standstill in normothermic laboratory animals following acute myocardial infarction.[14] The restoration of coordinated cardiac beating occurred in all of these animals with variable difficulty. Other investigators have shown that 15 per cent potassium chloride would promptly convert ventricular fibrillation to ventricular standstill in deeply hypothermic laboratory animals without acute myocardial infarctions[13]; in these studies restoration of cardiac beating was apparently accomplished with greater facility using concentrated Ringer's lactate and molar sodium lactate.

Interesting case reports in the recent literature give dramatic testimony to what may be accomplished with active resuscitation attempts.[2, 5, 6, 8, 12] We believe as others[1, 18] that many deaths from acute myocardial infarction are "electrical" rather than "muscular," and it is this belief, supported in part by earlier observations in the experimental animal, that provided the impetus for the present study.

The purpose of this paper is to report on the relative efficacy of 15 per cent potassium chloride and 30 per cent potassium citrate in converting ventricular

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Received for publication Dec. 22, 1958.

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fibrillation to ventricular standstill following acute myocardial infarction in the experimental animal. The ultimate aim of this effort is to standardize a procedure which can be instituted with sufficient ease and frequency to justify organized and vigorous efforts in attempts to resuscitate patients dying from coronary artery disease.

METHOD AND MATERIAL

Forty mongrel dogs weighing between 15 and 40 pounds were studied. The typical animal was lightly anesthetized with pentobarbital sodium and given positive pressure air and/or oxygen through an endotracheal tube; both mechanical respirators and bagging procedures were employed. Each experiment was performed utilizing strict aseptic technique. The chest was opened with a transsternal incision to enhance exposure. Hemostasis was carefully maintained. The femoral artery was cannulated for the withdrawal of blood specimens (pH, serum electrolyte, and chemical determinations) and the recording of arterial blood pressures. Continuous electrocardiograms and simultaneous arterial pressures were recorded on the Sanborn Twin Visocardiette. The femoral vein was cannulated for the administration of various drugs. The anterior descending branch of the left coronary artery was ligated with a 1-0 silk suture, 0.5 cm. distal to its bifurcation. Electrocardiographic tracings were followed until the changes characteristic of an acute myocardial infarction were observed. Subsequently, ventricular fibrillation either (a) occurred spontaneously within 10 to 15 minutes following the acute infarction or (b) was induced by electric shock (50 to 150 volts) applied directly to the heart, utilizing a defibrillator. Untreated ventricular fibrillation occurring under these circumstances was irreversible.

Immediately after the onset of ventricular fibrillation 20 animals were treated with 15 per cent potassium chloride to induce ventricular standstill and 20 animals were given 30 per cent potassium citrate. The effect of the intracardiac administration of hypertonic potassium (into one or both ventricles) was compared to the administration of the solutions into the proximal portion of the cross-clamped aorta and into one or both ventricles with the aorta cross-clamped.

Following the administration of potassium, slow cardiac massage (30 to 50 per minute) was initiated until the electrocardiogram demonstrated conversion of the ventricular fibrillation to ventricular standstill. The effectiveness of blood flow in the large arteries could roughly be gauged by the pressure recordings from the femoral artery. With the onset of ventricular standstill, cardiac message was speeded to 70 to 100 times per minute, and one molar sodium lactate was administered intravenously and occasionally in small amounts (up to 20 c.c.) into the heart. The total amount administered ranged from 30 to 350 c.c. (see Table I). In each animal studied, repeated episodes of ventricular fibrillation were terminated with hypertonic potassium chloride or potassium citrate, depending upon which agent was being studied

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with that particular animal. Restoration of cardiac beating following each episode of ventricular fibrillation in the same animal was approached, utilizing a method similar to that employed for the initial episode. Where cardiac message or spontaneous cardiac beating appeared to be unable to maintain a satisfactory level of arterial pressure, varying concentrations of intravenous l-norepinephrine were administered. Following successful restoration of cardiac beating and return of the blood pressure to near normal levels, the chest was closed with wire stay sutures and silk, and the animals were gradually weaned from the artificial respirator. The femoral catheters were subsequently removed, 10 to 15 minutes following the appearance of normal sinus rhythm associated with a normal or near normal arterial pressure. Postoperative animals were given intramuscular crystalline penicillin and streptomycin. No intravenous fluids were administered in the postoperative period; the animals were, however, allowed to eat and drink ad libitum. Frequent samples of blood for pH and serum electrolytes were analyzed using standard laboratory procedures to compare the electrolyte, chemical, and pH changes with 15 per cent potassium chloride and 30 per cent potassium citrate induced arrest.

RESULTS

Ventricular fibrillation, complicating acute myocardial infarction, in the experimental animal represents an irreversible terminal mechanism.

Changes in Arterial pH, Serum Electrolytes in Chemistry.—

1. *15 per cent potassium chloride:* Two 5 to 10 c.c. intracardiac injections of 15 per cent potassium chloride were given to terminate each episode of ventricular fibrillation. The total amount of potassium chloride administered was equally divided between the right and left ventricular cavity when intracardiac injection was studied. When the aorta was cross-clamped the total amount was given into the proximal portion of the aorta. It appears that conversion from ventricular fibrillation to ventricular standstill was accomplished with equal facility with both methods of administration. The total amount of potassium chloride required to induce ventricular standstill, following each episode of ventricular fibrillation, averaged 15 c.c. (see Table I).

Electrolyte and pH changes: The serum potassium concentrations attained their maximum immediately following the administration of potassium chloride. These very high levels (required to produce ventricular standstill) ranged from 8.4 mEq. per liter to 40.5 mEq. per liter. With the restoration of cardiac beating, high serum potassium levels fell precipitously, usually within 5 to 15 minutes, to levels ranging from 4.8 mEq. per liter to 10.8 mEq. per liter. As the effectiveness of cardiac beating improved, elevated serum potassium concentrations gradually returned to levels near normal or only slightly higher than normal (6.9 mEq. per liter).

Subsequent episodes of ventricular fibrillation, terminated with potassium chloride, were followed by an abrupt elevation in the serum potassium, usually to a concentration slightly more than that observed following termination of the initial episode. The serum chloride level showed a marked increase as a result of the hypertonic potassium chloride arrest (paralleling the serum potassium). The serum sodium concentration gradually increased following the intravenous administration of molar sodium lactate and reached its zenith toward the end of the experiment. Maximum serum sodium levels ranged from 138 mEq. per liter to 216 mEq. per liter. The serum calcium level decreased slightly throughout the experiment; the low range being 8.2 mg. per cent to 8.7 mg. per cent. The carbon dioxide combining power decreased abruptly following induced cardiac arrest (Fig. 1*B*) and subsequently returned to near normal levels. The average fall was 14 mEq. per liter. There was no significant change in the serum magnesium concentration. The arterial pH fell significantly. The most abrupt fall occurred immediately after the administration of potassium chloride and in general, coincided with the onset of ventricular standstill. Subsequently, the blood pH either remained low, in spite of the administration of molar sodium lactate, or was decreased further by an unknown, apparently paradoxical, mechanism. The average fall of the arterial pH was 0.32. Fig. 1*A* and 1*B* show the significant mean changes in the serum sodium, potassium, carbon dioxide combining power and arterial pH occurring during the procedure.

2. *30 per cent potassium citrate:* In 20 animals studied with 30 per cent potassium citrate arrest, the most effective method of administration appeared to be equal division of the total amount into both the right and left ventricle. The total amount of potassium citrate required to induce ventricular arrest (standstill) ranged from 4 c.c. to 32 c.c.

Electrolyte and pH changes: In general, the changes in the serum electrolytes corresponded to those observed following hypertonic potassium chloride with several exceptions. The serum chloride level remained essentially unchanged following potassium citrate arrest, and the increase in the serum potassium level was never as marked as that observed following potassium chloride arrest. The maximum serum potassium concentrations ranged between 10.1 and 19.5 mEq. per liter. The carbon dioxide combining power decreased slightly throughout the procedure (as with potassium chloride), and the serum calcium either remained unchanged or decreased slightly, following, and probably as a result of, the intravenous sodium lactate therapy.[4] There was no apparent change in the serum magnesium. Comparative mean electrolyte and pH changes are shown in Figs. 1*A* and 1*B*.

Effect of 15 Per Cent Potassium Chloride and 30 Per Cent Potassium Citrate on Resuscitation From Ventricular Fibrillation.—

The mean number of episodes of ventricular fibrillation using hypertonic potassium chloride arrest was 5; with hypertonic potassium citrate arrest, this was reduced to 3.

In 39 of the 40 animals studied, the intracardiac administration of both hypertonic potassium solutions converted the ventricular fibrillation to ventricular standstill; one animal died an anesthetic death. In general, hypertonic potassium citrate appeared to be more effective in that ventricular

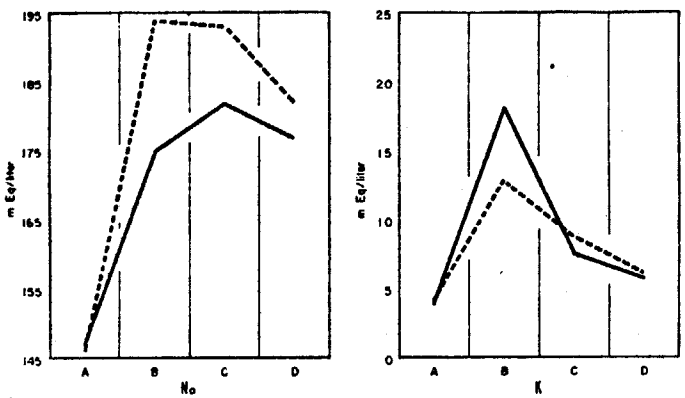

Fig. 1A.— ——— K Cl 15%. ----- K citrate 30%. *A*, Before opening chest. *B*, Cardiac arrest. *C*, Restoration of cardiac action. *D*, End of procedure.

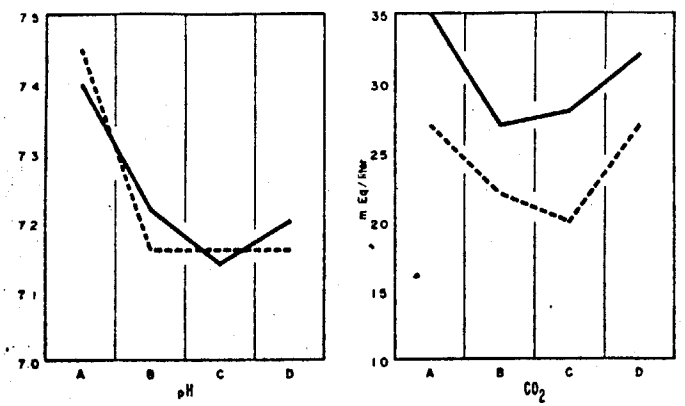

Fig. 1B.— ——— K Cl 15%. ----- K citrate 30%. *A*, Before opening chest. *B*, Cardiac arrest. *C*, Restoration of cardiac action. *D*, End of procedure.

standstill occurred more promptly and with greater facility; the tendency to develop multiple episodes of ventricular fibrillation was reduced with 30 per cent potassium citrate (Table I).

In 19 of the 40 animals, we were able to restore the heart to normal sinus rhythm and the blood pressure to normal or near normal ranges (Fig. 2).

TABLE I

TYPE OF K SOLUTION USED	AVERAGE AMOUNT K TO TERMINATE EACH EPISODE OF VENTRICULAR FIBRILLATION	AVERAGE NO. OF EPISODES OF VENTRICULAR FILBRILLATION	AVERAGE DURATION OF INITIAL EPISODE OF VENTRICULAR FIBRILLATION	AVERAGE AMOUNT MSL* AFTER EACH EPISODE OF ARREST
15% KCl	15 c.c.	5	3 min.	100 c.c.
30% K Citrate	5 c.c.	3	50 sec.	40 c.c.

*Molar sodium lactate.

Twelve of these subsequently died while the chest was being closed. Six of the 12 animals died as a result of the following difficulties: hemorrhage (1 dog); apparently irreversible damage to the respiratory center (manifested by an inability to resume breathing spontaneously [2 dogs]); trauma and laceration of the myocardium incident to massage (2 dogs); and inadequate pulmonary ventilation following closure of the chest (1 dog). Seven animals survived the entire procedure, living from 1 hour to 6 months. The hearts of these animals were subsequently examined grossly and microscopically.

In the remaining 20 animals, normal sinus rhythm never became re-established. In many of these, an auriculoventricular nodal rhythm or a transient return to normal sinus rhythm was observed, following which repeated episodes of ventricular fibrillation signified the end of the experiment.

Pathologic Examination of the Heart.—

Pathologic examination of the hearts at varying periods of time following successful cardiac resuscitation showed either the change of an acute or chronic myocardial infarction. In an animal sacrificed after surviving 6 months, the large anterolateral myocardial infarction visualized during the acute procedure had healed so that only the residual changes of a small transmural area of fibrosis in the anterior wall of the left ventricular myocardium was demonstrated histologically.

DISCUSSION

Previous experimental work had demonstrated that in spite of an acute, apparently extensive, myocardial infarction, 3 out of 4 animals studied with terminal ventricular fibrillation were ultimately restored to a supraventricular rhythm with normal or near normal blood pressures.[14] In the present study, performed utilizing strict aseptic technique, earlier observations (using hypertonic potassium chloride) were repeated and compared with hypertonic potassium citrate arrest. From this study it appears that 30 per cent potassium citrate is superior to 15 per cent potassium chloride in converting ventricular fibrillation to cardiac standstill. Induced potassium citrate arrest resulted in less severe potassium intoxication which appeared to be more readily reversed by treatment with molar sodium lactate.[3] Apparently undesirable hyperchloremia observed with potassium chloride was not present when potassium

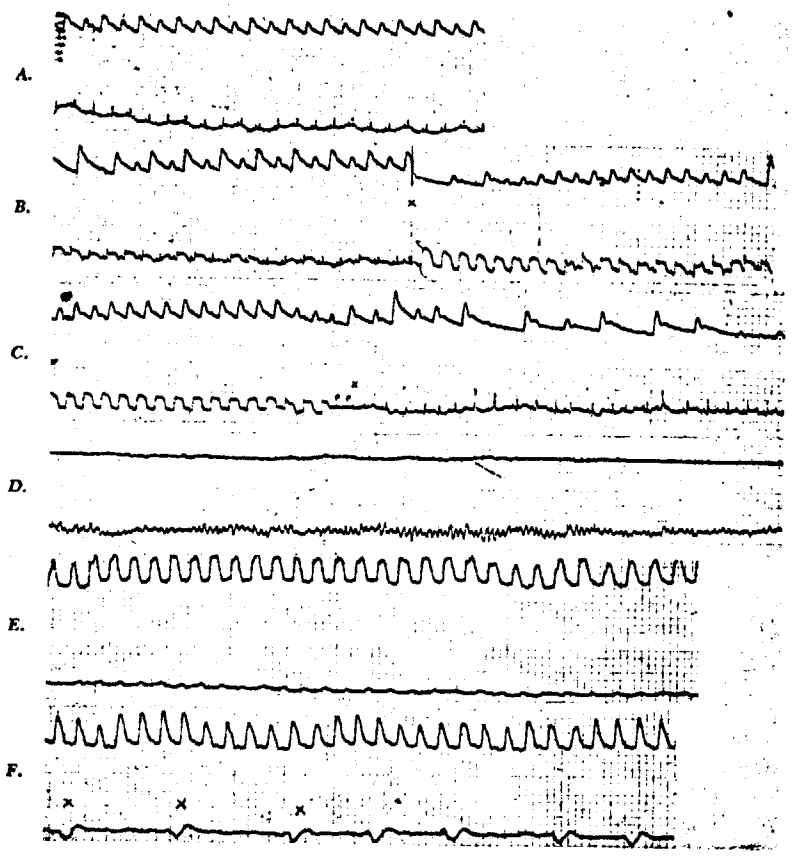

Fig. 2.—*A*, Control (Lead II); simultaneous arterial pressure is 120/80 mm. Hg.

B, First half of record taken 10 minutes after ligation of the anterior descending coronary artery shows RS-T segment elevation of the type seen in acute coronary artery occlusion. The arterial pressure is 124/60 mm. Hg. From *X* until the end of the record, note the characteristic high take-off of the RS-T segment accompanying the acute myocardial infarction. The pressure has fallen to 84/44 mm. Hg.

C, Thirty minutes after acute coronary ligation note the conduction disturbance at *X*.

D, Forty minutes following acute coronary artery occlusion at 2.30 P.M., ventricular fibrillation was induced by applying electrodes to the ventricles and administering a single 50-volt electric shock.

E, Forty-five seconds after 3 c.c. of 30 per cent potassium citrate had been given into both ventricles; ventricular fibrillation has been converted to ventricular standstill. Cardiac massage was started (at a rate of 100 per minute). Note the apparent effectiveness of arterial flow as recorded by a catheter in the femoral artery.

F, Three minutes after ventricular standstill, note the onset of electrical activity at *X* with widened, bizarre QRS complexes. Intravenous molar sodium lactate has been started at a rate of 5 c.c. per minute.

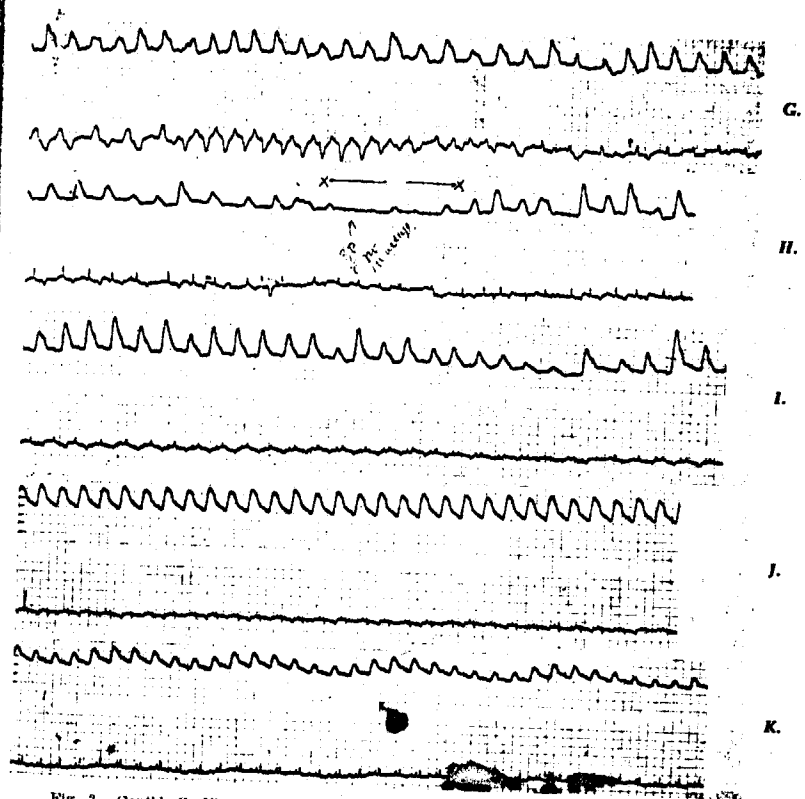

Fig. 2.—Cont'd. *G*, Five minutes after *F*, following a total of 40 c.c. of molar sodium lactate, there is progressive narrowing of the QRS complexes and a suggestion of restoration of auricular activity (*P*). Cardiac massage has been continuous.

H. Twelve minutes after ventricular standstill, the QRS complexes have narrowed to a near normal width. Note the effects (*X*) on the arterial pressure of discontinuing cardiac massage.

I, Fifteen minutes after induced ventricular arrest, there is a return to normal sinus rhythm. The arterial pressure is being supported with a very slow intravenous drip containing 1 ampule of l-norepinephrine diluted in 40 c.c. molar sodium lactate.

J, Twenty-five minutes after ventricular standstill, there is spontaneous effective cardiac beating (without massage). The arterial pressure is maintained at 110/60 mm. Hg without massage or drugs. The QRS complexes are low in amplitude. The T-wave is inverted and the QT interval appears prolonged for the heart rate (approximately 116 per minute).

K, One hour and thirty minutes after "death" in ventricular fibrillation, the arterial pressure is 100/60 mm. Hg. The chest has been closed and the animal has resumed spontaneous breathing.

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citrate was used. The paradoxical observation that the arterial pH continued to remain low with both hypertonic potassium solutions, even after restoration of effective cardiac beating, is difficult to understand; particularly after rather large amounts of a mild alkalizing solution (sodium lactate) had been given. In the hope of clarifying this, we are at the present time studying the carbon dioxide tension in the plasma.

Changes in the serum calcium probably reflected the use of varying amounts of sodium lactate intravenously as has been previously noted.[4] This decrease in the serum calcium would appear to increase the cardiotoxic effect of even a slight elevation in the serum potassium concentration, and we should like to be able to prevent this. Earlier studies have suggested that calcium is useful in hearts arrested by potassium.[9, 16] In our experience, however, it seemed difficult to quantitate the amount of calcium which was helpful and the danger of overdosage (recurrent ventricular fibrillation) limited its usage.

Blood Pressure Changes.—The persistent acidosis might explain the tendency for some of the animals to remain mildly hypotensive even following the return of a normal sinus mechanism (electrocardiographically) with apparently forceful, effective, mechanical cardiac contractions. In these animals, the combination of l-norepinephrine and molar sodium lactate often successfully restored the blood pressure to normal or near normal levels. One might anticipate such a reaction as a result of the observation that alkalosis appears to enhance the effectiveness of intravenous vasopressor agents.[7]

In the animal surviving longest (6 months), an interesting, perhaps superfluous, observation is the fact that this dog delivered a litter of 4 apparently normal pups approximately 5 months after successful cardiac resuscitation.

From our observations it appears that one can anticipate occasional long-term survival animals with a standardized resuscitation attempt utilizing 30 per cent potassium citrate to induce ventricular arrest and a combination of cardiac massage, molar sodium lactate, and l-norepinephrine. Further studies have been initiated to elaborate on these observations.

SUMMARY

Thirty per cent potassium citrate was more effective than hypertonic potassium chloride in converting otherwise irreversible ventricular fibrillation to ventricular standstill in normothermic laboratory animals with acute myocardial infarction. Continuous electrocardiograms and arterial pressures were recorded throughout each procedure and serial electrolyte, chemical, and pH changes were studied and are discussed.

Of the 40 animals included in this report, 7 survived and were observed from hours to months following successful cardiac resuscitation.

Some of the problems encountered in this study are presented. The need for further experimental observations in organized resuscitative attempts following death from acute myocardial infarction is stressed.

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DETECTION OF TRIETHYL CITRATE AND TRIACETIN IN LIQUID AND DRIED EGG WHITES[1]

L. KOGAN AND S. STREZLECK

ABSTRACT

A method is described for detection and identification of the whipping aids triethyl citrate and triacetin in egg whites. The sample, either liquid whites or dried whites that have been reconstituted, is extracted with diethyl ether. The presence of the whipping aid in the residue of the ether extract is established by gas-liquid chromatography. Unequivocal identification is made by infrared spectroscopy, using the gas-chromatographically purified material to obtain the infrared spectrum.

Triethyl citrate (1) and triacetin (2) are additives used in egg whites to improve the whipping properties. When added to liquid or dried egg whites, these compounds markedly decrease the time required for whipping the egg whites to a uniform, stable foam. Normal use levels for these additives are quite low (about 0.025% for triethyl citrate and about 0.15% for triacetin, both on a liquid egg white basis). The procedure described herein was developed to fill a need for a more reliable means of detecting and identifying these egg white additives. Prior to the development of this procedure, these additives were identified after extraction from the egg white with diethyl ether by simple qualitative tests. Triethyl citrate was characterized as an ester by the hydroxamic acid test. In this test, the ester is converted into the sodium salt of the corresponding hydroxamic acid by reaction with hydroxylamine hydrochloride in the presence of sodium hydroxide. This in turn reacts with ferric chloride in the presence of acid to produce a red or violet-colored ferric salt. The ester is further identified as being derived from citric acid by the citrizinic acid test (the formation of a fluorescent blue ammonium salt of citrizinic acid). The correct refractive index and exhibition of the characteristic bitter taste completed the identification. Similarly, triacetin was identified by the ester test, a qualitative test for glycerol (acrolein test), by refractive index, and by checking for the acetic acid moiety after hydrolysis.

Materials and Methods

Gas-Liquid Chromatography. The instrument used was the Chromacon, model 9475-35 (made by Podbielniak, Inc.) with thermal con-

[1]Manuscript received September 2, 1965. Contribution from The Fleischmann Laboratories, Standard Brands Inc., Betts Ave., Stamford, Conn.

ductivity detector. The instrument was modified so that the detector could be operated with a bridge current of 200–300 milliamp., thus greatly increasing the sensitivity. (The instrument was designed to operate with a bridge current of 50 milliamp.) A heater was installed around the column outlet to prevent excessive premature condensation of the vapors eluted from the column in this portion of the apparatus. This facilitates collection of the purified condensed triethyl citrate and triacetin vapors in the collection bottle. Condensation of the eluted vapors in the column outlet prior to collection of the purified sample is undesirable, since it would permit contamination of the sample and defeat the purpose of the gas-chromatographic purification.

The column used was 1/4-in., 6-ft. copper tubing packed with Chromosorb-W (30/60-mesh) coated with silicone gum rubber (SE-30). To prepare the column packing, 20 g. of the silicone gum rubber was dissolved in sufficient chloroform to cover 100 g. of the Chromosorb. After thorough mixing, the chloroform was evaporated off on a steam bath before the column was packed.

Operating temperatures were (°C.): sample injection block, 250; column, 200; column outlet heater, 165.

The carrier gas was helium at a pressure of 10 p.s.i.

Spectroscopic Measurements. Infrared spectral identifications were obtained on thin films (on salt plates) of the purified material collected at the column outlet of the gas chromatograph. The instrument used was the Perkin-Elmer, model 137.

Detection of Triethyl Citrate and Triacetin. Extraction. The liquid egg whites were extracted with diethyl ether (dried egg whites were reconstituted at a ratio of 7.15 g. to 50 ml. of water prior to the ether extraction). Triethyl citrate is more readily extracted if the pH of the liquid whites is adjusted to 3.0 with concentrated H_2SO_4 prior to extraction. Because triacetin hydrolyzes readily at pH 3 to yield glycerol and acetic acid, ether extraction of this additive must be made on unacidified egg white. This means that two extractions must be carried out on unknown egg white samples: 1) on the acidified sample to determine triethyl citrate, and 2) on the unacidified sample to determine triacetin.

It is usually necessary to extract 500 ml. of liquid egg white to obtain sufficient material to complete the identification. About 1 liter of diethyl ether was required for the extraction, which was carried out in a large separatory funnel. Successive 100-ml. portions of the liquid egg white were shaken with the same 1-liter portion of ether. This was accomplished by drawing off most of the first 100 ml. of sample after it was shaken with the ether. The second 100 ml. was then

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introduced into the separatory funnel, shaken, and allowed to separate; the egg white layer was then drawn off and discarded. This process was repeated until the entire 500 ml. of sample had been extracted. The ether layer was filtered through glass wool to separate any remaining albumen. The glass wool was washed with additional diethyl ether. The ether used to wash the glass wool was combined with the ether extract of the sample and concentrated by evaporation to about 1 ml. under a stream of nitrogen. Presumptive evidence of the presence of triethyl citrate or triacetin was obtained by gas chromatography of 5 μl. of this concentrated extract. Under the above operating conditions, the peak for triacetin appeared after about 8 min. and that for triethyl citrate after 23 min.

Purification of Extract and IR Identification. If a positive presumptive test was obtained, the remainder of the ether extract was evaporated under a stream of nitrogen until all of the ether was removed. Twenty-five microliters of the oily residue was placed on the column. When the recorder began to trace the peak associated with the additive in question, a small vial was placed over the outlet of the column and the condensing vapors were collected. To cool the vial slightly, it was wrapped with a wet cloth. The condensate was collected as long as the recorder continued to trace the peak. Infrared spectra were obtained by dissolving the condensate in a small volume of anhydrous diethyl ether and transferring a thin film to the salt crystal by evaporating a drop of the solution on the crystal.

Results and Discussion

The procedure was tested by adding triethyl citrate and triacetin at use levels to separate samples of liquid egg white. After extraction and gas-chromatographic purification, characteristic IR spectra were obtained for triethyl citrate and triacetin. Figure 1 shows the IR spectrum of triethyl citrate and of the triethyl citrate isolated from the egg white. The IR spectra for triacetin and for the triacetin extracted from the egg white sample are shown in Fig. 2.

A commercial sample of dried egg white known to contain triacetin was examined by the procedure described. The preliminary gas chromatography indicated that the sample contained triacetin plus an impurity. The IR spectrum for the purified extract was similar to that for triacetin but slightly different. When the column temperature was reduced to 150°C., the triacetin separated sufficiently from the impurity to be collected. Infrared spectroscopy of this purified material gave a characteristic spectrum for triacetin. The retention time for the impurity indicated that it was probably diacetin. Since the peak for

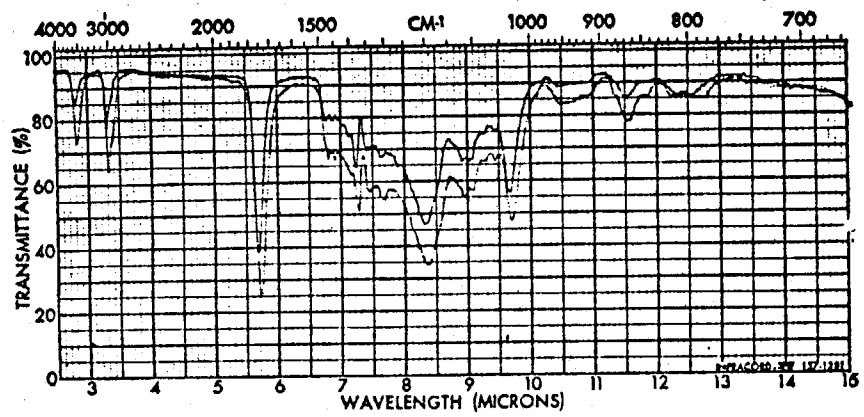

Fig. 1. Upper curve, infrared spectrum of triethyl citrate extracted from egg white; lower curve, infrared spectrum of triethyl citrate.

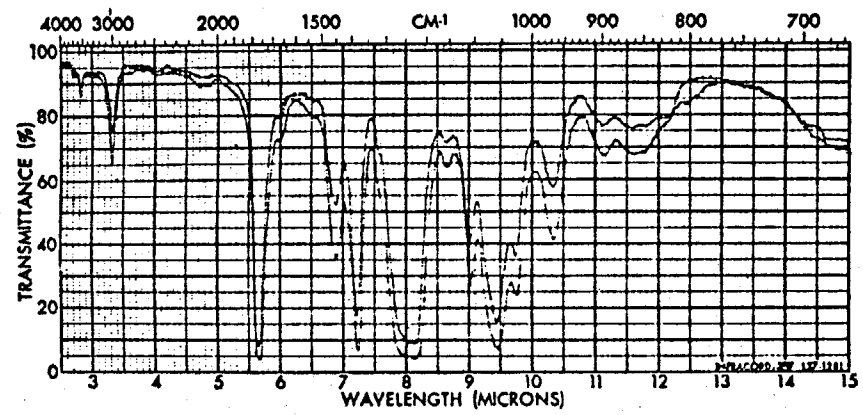

Fig. 2. Upper curve, infrared spectrum of triacetin extracted from egg white; lower curve, infrared spectrum of tracetin.

this impurity appeared before the triacetin peak and since it was present at a very low level relative to the triacetin, no further effort was made to confirm the identity of the impurity.

The procedure is recommended only for its qualitative applications. No recovery experiments were carried out during the development of this procedure, because our experience with the extraction of liquid and reconstituted dried egg whites for colorimetric assays by the hydroxamic acid test has shown that only about 50 to 85% recoveries of triacetin and triethyl citrate may be expected when quantities as large as those required for this test are extracted. We have, however, experienced no difficulty in detecting these additives by this

procedure when present at effective use levels. In our experience as little as 0.2 to 0.3 mg. of triethyl citrate or triacetin is ample to obtain infrared spectra similar to those shown in Figs. 1 and 2. The recoverable amounts of triacetin and/or triethyl citrate extracted from the egg whites containing use levels of the respective additives are greatly in excess of the IR requirements.

Acknowledgment

The authors express their appreciation to Edward J. Healy for technical assistance in the gas-chromatographic and infrared examinations and to Wayne E. Maynard for his helpful criticism in the preparation of this paper.

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Toxicity of Plastics Used in Medical Practice II

Toxicity of Citric Acid Esters Used as Plasticizers

By D. B. MEYERS, J. AUTIAN, and W. L. GUESS

The acute and chronic toxicity of triethyl citrate, acetyl triethyl citrate, tributyl citrate, and acetyl tributyl citrate was studied in a series of small animals. The behavior response to parenteral administration of the esters was observed in rats, mice, frogs, and rabbits. Mice were used to determine the LD_{50} for each compound. All of the esters exhibited a marked effect on the CNS evidenced by increased respiration rate, convulsions, and cord depression. When applied locally to the nerve, the citrates rapidly blocked nerve conduction. A profound depressor action was observed in cats and rabbits which was attributed to a direct cardiac inhibitory effect. Chronic administration for a period of 2 weeks resulted in a depression of weight gain; in the animals that received acetyl tributyl citrate evidence of a change in the blood picture was observed. Histopathological examinations revealed no damage to the liver, kidney, lungs, and spinal cord in the chronic animals.

MUCH PROGRESS has been made in the use of plastics for various devices used in medicine and pharmacy in the last 5 years. Advancements in plastic technology have made possible many improvements in existing devices which would not have been possible with more conventional materials. However, certain problems have been noted which indicate that greater care should be taken in selecting the plastic device (1–3).

Tubings for the administration of drug and blood products or for the collection of body fluids may be composed of one or more polymeric materials. Usually the polymer is of the vinyl type, if softness, flexibility, and clarity are desired. Plasticizers and other additives must be added to this polymeric material to give the final device the desired physical and chemical properties. Thus, it is possible to have a host of plastic formulas, referred to as polyvinyl chloride, but each differing from the other in composition. In most instances the various ingredients used for polyvinyl chloride tubings which are to be used in medical practice have been approved as food additives by the FDA.

In 1960, Autian and Kapadia (4) reported that certain polyvinyl chloride tubings released a constituent to several hydroalcoholic solvent systems. They did not, however, investigate the possible biological effect of the leached constituent. Meyler, Willebrands, and Durrer (5) in the same year reported that certain polyvinyl chloride tubings released a constituent to blood which in turn caused cardiac arrest and ventricular fibrillation on the isolated perfused heart of rats. Keith, *et al.* (6), reported that one lot of plastic tubings (vinyl type) appeared to enhance the hemolytic effect on perfused blood. This observation was further substantiated with *in vitro* studies by Hirose and associates (7). Lawrence, *et al.* (8), noted that a number of commercially available polyvinyl chloride tubings used as part of administration devices released a constituent into tissue causing a toxic response when implanted for short periods of time in animals. In this particular study the offending agent in the plastic was not the plasticizer but one of the other additives.

Even though the work of Lawrence, *et al.* revealed that the plasticizers were not causing adverse effects by the experimental method employed, there is no assurance that the same plasticizers or other plasticizers will not produce a biological effect when administered in a different manner to animals. Since present vinyl tubings contain a large proportion of one or more plasticizers and since these tubings will have contact with drug systems or body tissues for varying periods of time, it was thought prudent to investigate the pharmacological properties of one series of plasticizers (citric acid esters) which had previously been reported as quite safe by oral feeding (9).

EXPERIMENTAL

The compounds used in this study included triethyl citrate (TEC), acetyl triethyl citrate (ATEC), tributyl citrate (TBC), and acetyl tributyl citrate (ATBC). The esters were commercial samples containing less than 1% foreign material.[1] Because of the nearly insoluble nature of the butyl

Received June 3, 1963, from the Drug-Plastic Research Laboratory, College of Pharmacy, University of Texas, Austin.
Accepted for publication October 2, 1963.
The authors gratefully acknowledge Mr. S. A. Rosenbluth for his technical assistance and Dr. S. W. Bohls and associates, Austin State Hospital, for the histopathology studies.
This investigation was supported by Grant CA-06120 from the National Cancer Institute, U. S. Public Health Service, Bethesda, Md.
Presented to the Scientific Section, A.PH.A., Miami Beach meeting, May 1963.

[1] Samples supplied by Charles Pfizer and Co., Inc., Brooklyn, N. Y.

derivatives, all the esters were administered suspended or dissolved in 3% acacia throughout the experimental work. Ester concentration was varied to be suitable for each particular study.

Behavior Studies.—The general overt effects of the esters were first observed following i.p. administration of graded doses into Swiss albino mice. Both of the ethyl citrates produced a very rapid loss of righting reflex without loss of consciousness in doses slightly exceeding 400 mg./Kg. Usually the animals regained their posture within 15 minutes. Respiration rate was markedly increased and frequently clonic convulsions were observed. The butyl derivatives failed to produce the rapid loss of righting reflex, but did evoke an increase in respiration which was frequently accompanied by clonic convulsions. Some writhing was observed during the first 10 minutes after injection of the butyl compounds. All of the esters produced the same signs of stimulation in Wistar rats when given i.p. with both TEC and ATEC, again causing a rapid loss of righting reflex of short duration when the dosage exceeded 400 mg./Kg. The stimulating properties of the esters were confirmed by intravenous administration into rabbits. Marked increases in motor activity and respiration were observed following doses of 100 mg./Kg.

Doses of 1000 mg./Kg. placed in the ventral lymph sac of the frog resulted in a short period of clonic activity, followed by the complete abolishment of all reflex activity.

Acute Toxicity.—The i.p. LD_{50}'s for the esters were determined in Swiss albino mice weighing between 16 and 20 Gm. Doses were chosen from a logarithmic scale and the median lethal dose calculated by method of Bliss (10). All deaths occurred in the first hour following administration of TEC and ATEC, but in the case of the butyl compounds a 72-hour observation period was required. The LD_{50} with standard error for TEC was 1750 ± 68 mg./Kg.; for ATEC, 1150 ± 185 mg./Kg.; for TBC, 2900 ± 210 mg./Kg.; and for ATBC, >4000 mg./Kg. Death was attributed to circulatory collapse and postictal depression.

Chronic Toxicity Studies.—To study cumulative effects of the citrates, a 14-day chronic toxicity test was carried out in which mice weighing from 16–20 Gm. were given daily i.p. doses which approximated one-fifth of the acute median lethal dose. Five groups of 20 mice each were selected and the weight, RBC count, WBC count, clotting time, and hemoglobin level of each individual determined. Group *A* received daily injections of TEC 350 mg./Kg.; Group *B*, 230 mg./Kg. of ATEC; Group *C*, 580 mg./Kg. of TBC; and Group *D*, 900 mg./Kg. of ATBC. Group *E* received daily injections of the 3% acacia vehicle and served as the control. The animals were weighed daily and at the end of the 2-week period the RBC count, WBC count, clotting time, and hemoglobin level were redetermined.

Figure 1 shows that all of the compounds inhibited normal weight gain. At the 7-day period this inhibition was significant within 95% confidence limits. It was most striking in the case of ATBC which, although given in the largest doses, had exhibited the lowest acute toxicity.

There were no significant differences in the RBC counts, the WBC counts, the clotting times, and the hemoglobin levels in the mice that received TEC, ATEC, or TBC. However, significant (95% confidence) fall in the RBC count and hemoglobin levels in those animals administered ATBC occurred.

In a further study of the blood picture changes due to ATBC, two albino rabbits were administered 450 mg./Kg. of ATBC daily for 14 days. Two others were given 900 mg./Kg. daily for 7 days. At the end of the period, the RBC count, hemoglobin level, and a differential WBC count were made; red bone marrow smears from the femur bone were examined. In all animals there was a fall in the red blood cell count ranging from 0.5 to 2.5 million and a corresponding fall in hemoglobin levels. However, the basophil count and the bone marrow smears gave no indication of aplastic anemia.

At the end of the chronic toxicity study, two mice from each group were sacrificed; slides were made of liver, lung, and kidney tissues. Examination of these slides did not reveal pathological cellular changes in these organs.

On the day following withdrawal from the chronic test, hexobarbital sleeping time studies were conducted in the remaining mice. There was no significant difference in the sleeping time within the various groups. This confirmed that liver function was not impaired by chronic administration of the esters.

Effects of the Esters on Neural Muscular Transmission.—Behavior studies indicated that the citrate esters produced some neurological effects. Therefore, a series of studies was set up to determine the nature of the neural involvement. In the first experiment, white rats were anesthetized with pentobarbital, and right anterior tibialis muscle freed and arranged for recording contractions, and shielded electrodes placed central to a pledget of cotton around the sciatic nerve. Stimulus was supplied through the electrodes by a stimulator set to deliver a current which would produce a submaximal response every 10 seconds. After obtaining a normal response, 3 drops of the ester were placed on the cotton. All four esters produced a complete sciatic nerve block which was reversible when the cotton was removed and the nerve washed with saline. This activity was confirmed using the isolated gastrocnemius muscle-sciatic nerve preparation from the frog.

To determine central nervous system involvement, pentobarbital anesthetized white rats were set up so that the contralateral reflex could be recorded. After freeing the anterior tibialis muscle in the left leg and arranging it for recording contractions, shielded electrodes were placed around

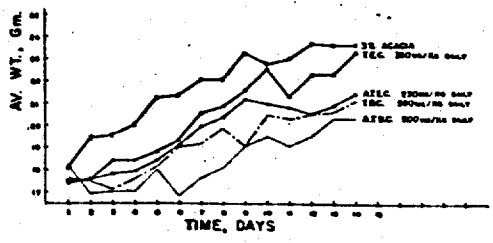

Fig. 1.—The effect of some citric acid esters administered i.p. on weight gain of mice.

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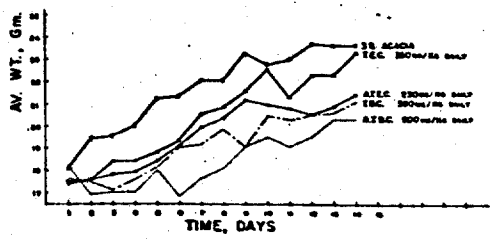

Fig. 1.—The effect of some citric acid esters administered i.p. on weight gain of mice.

the sciatic nerve in the right leg. The stimulator was set to deliver a series of five impulses 10 seconds apart when activated by a switch. After determining a normal submaximal contralateral reflex, each ester was administered through the jugular vein which had previously been cannulated. Figure 2 shows that all of the esters were capable of completely blocking this reflex. The duration of blockage varied according to the ester. Considerably larger doses of ATBC were required to affect the block, but the duration was much longer. In contrast, TBC block occurred in response to the lowest dose but lasted longer than that produced by either of the ethyl esters.

To verify that cord depression and not myoneural junction blocking was the cause of the inhibited contralateral reflex, rats were prepared so that the sciatic nerve stimulation and anterior tibialis muscle contraction occurred on the same side. Doses of the esters which were twice as great as those which abolished the contralateral reflex failed to impair myoneural junction transmission.

Local Anesthesia Action.—All four of the esters temporarily abolished the corneal reflex when 3 drops of a 5% suspension were placed in the conjunctival sac of the rabbit. As expected, the butyl esters were longer in duration than their ethyl counterparts. This local anesthetic effect was confirmed by the intradermal administration of

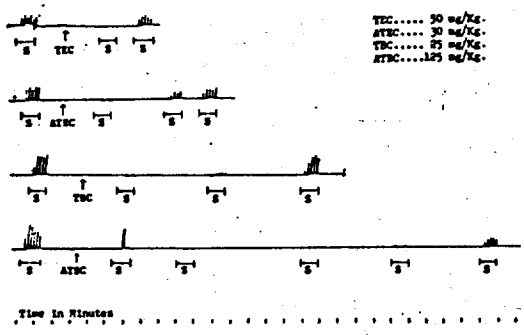

Fig. 2.—Effect of some citric acid esters administered i.v. on the contralateral reflex of the rat.

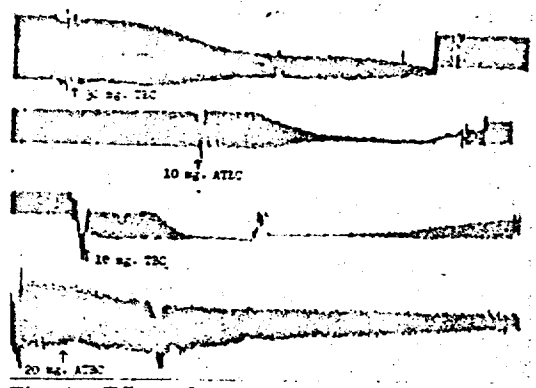

Fig. 4.—Effect of some citric acid esters on rate and amplitude of the isolated rabbit heart.

0.1 ml. of a 2% suspension of the esters into shaved areas on the back of guinea pigs. The duration of insensitivity to pricking of the surrounding area with a pin was 12 to 20 minutes for TEC. However, TBC produced a deadened area for a period of over 2 hours.

Cardiovascular Action of the Citrate Esters.—Acute toxicity studies indicated probable cardiovascular collapse, so a study of the effect of the citrate esters on blood pressure was indicated. Rabbits and cats were used in this study. Blood pressures were recorded from the cannulated carotid artery with a mercury manometer. A tambour connected to the tracheal cannula simultaneously recorded respiration. The esters were administered through the ear vein or through the cannulated jugular. Figure 3 is a tracing showing the typical responses obtained from varying doses of the esters in rabbits. There was an apparent dose-response relationship with all the compounds, and all of them produced complete loss of blood pressure when administered in toxic doses. Interestingly, the comparative doses required to produce a fall in blood pressure for the esters were quite well related to those which were required to produce death. ATBC with a high LD_{50} (greater than 4000 mg./Kg.) did not produce a significant fall in blood pressure in low

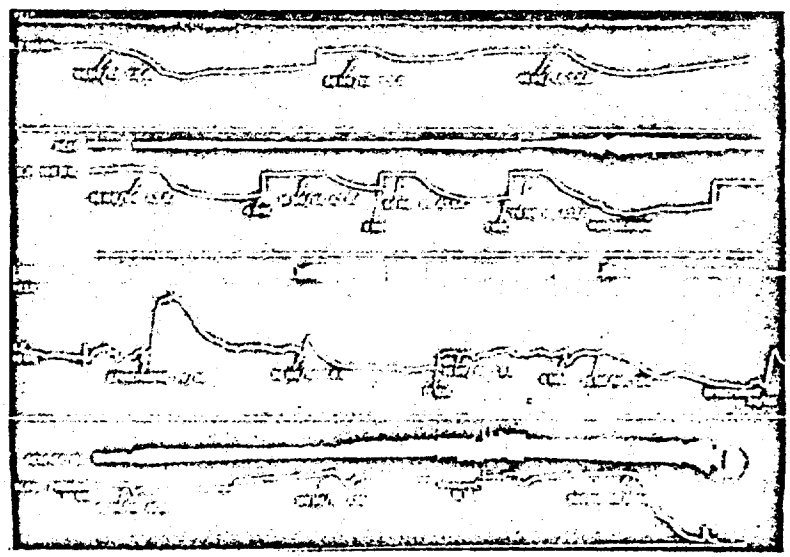

Fig. 3.—Influence of some citric acid esters on blood pressure and respiration of rabbits under pentobarbital anesthesia.

doses. The depressor effect of the esters was similar in cats and rabbits. It could not be blocked by atropine, and it could be reversed by epinephrine. This was indicative that the effect was due to direct smooth muscle relaxation or to the cardiac depression which was quite apparent.

To show that the depressor effect was primarily due to cardiac inhibition, the esters were tested on the isolated rabbit heart after the method of Langendorff (11). As can be seen in Fig. 4, all of the compounds brought about a sharp decline in both rate and amplitude of the heart beat. If given in carefully controlled doses, the heart returned to normal, but higher doses resulted in complete heart arrest. Coronary flow was also markedly diminished, precluding the possibility of significant direct smooth muscle relaxation.

SUMMARY AND CONCLUSIONS

The results of this study indicate that the four citric acid esters used as plasticizers have well defined and marked pharmacological activity when administered parenterally. All four have local anesthetic action and can block neural transmission when they come in direct contact with a nerve trunk. All of them diffuse from the blood stream into the cord in sufficient concentration to depress cord function. At the same time they apparently stimulate the higher centers. Although they vary widely in potency, both the ethyl esters and the butyl esters depress cardiac activity sufficiently to produce cardiovascular collapse. Chronic administration of three of them (TEC, ATEC, and TBC) does not appear to have an effect on the blood picture, but there are indications that ATBC may inhibit production or increase destruction of red blood cells. No species variation was observed throughout the investigation.

One must take care not to extrapolate the results reported in this paper to actual polyvinyl chloride devices which might contain plasticizers as reported here, since the biological activity will be elicited only when the plasticizer is released into the body fluids and, further, that the magnitude of the response shall have a direct relation to the concentration released. The biological responses reported here for a group of plasticizers should, however, alert those manufacturing plastic devices to learn more about the ingredients they employ in their plastic formulation if these items are to be introduced into medical practice.

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Rheology of Gelatin Films

By ROBERT A. CASTELLO* and JERE E. GOYAN‡

A procedure has been developed for the evaluation of the viscoelastic properties of gelatin films employed in soft gelatin capsule formulations. It involves the measurement of the tensile relaxation modulus of the film and the fitting of the resultant curve on an analog computer to obtain the appropriate constants. A theory was proposed for the mechanism of stress relaxation in gelatin films. Based upon this theory, an equation for the tensile relaxation modulus was derived. This equation was of the same form as the empirical equation which fit the stress relaxation curves of the gelatin films.

Although much information has been gathered concerning changes in the rheological properties of gelatin solutions upon thermal aging, little is known of the characteristics of gelatin films. Since the rheological behavior of gelatin films influences the properties of capsules molded from them, information concerning changes in this behavior might be helpful in evaluating technological problems encountered in the encapsulation process.

The viscoelastic properties of gelatin have been investigated by several authors. Among them, Ferry (1, 2) has studied the concentration effect on gel rigidities, and Tobolsky (3, 4) has studied stress relaxation as a tool in the investigation of gelation mechanisms. In this study the stress relaxation model has been

Received June 3, 1963, from the College of Pharmacy, University of Michigan, Ann Arbor.
Accepted for publication October 7, 1963.
Abstracted from a thesis submitted by Robert A. Castello to the Horace H. Rackham School of Graduate Studies in partial fulfillment of Doctor of Philosophy degree requirements.
Presented to the Scientific Section, A.Ph.A., Miami Beach meeting, May 1963.
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On the natural occurrence of ethyl esters of citric acid

by G. Neurath and W. Luettich

Report from the Central Laboratory of H.F. & Ph. F. Reemtsma, Hamburg

(Received on July 14, 1967)

Despite the wide-spread use of citric acid as a produce of metabolism in the vegetable and animal kingdom and the high concentrations in which it is found, for example,5 to 7% in lemon juice, little is known so far about the occurrence of the esters of citric acid. Only the citric acid trimethyl ester and the symmetrical citric acid dimethyl ester (citric acid-alpha-alpha'-dimethyl ester) were found by Bose and Bhattacharyya (1) in a study of the year 1936 in "Ananas sativus." Pinoow described in 1915 the isolation of a citric acid ethyl ester from fermented lemon juice, without giving it a name (2) *.

It seemed therefore of interest to study this field again, using modern separation- and identificationmethods, particularly since citric acid and its esters are widely used today in food technology, for example, as combination partners with antioxidants (synergists) (3,4), where their effect consists in inactivating metal-ions, and as plasticizers (5).

For the analytical proof of polycarboxylic acid-alkyl esters, no suitable generally applicable method has been described so far in the literature. While the neutral esters can be readily isolated by gas chromatography, the gas chromatography of the esters with free carboxyl groups is possible only after methylation, that is, after they have been converted to natural esters. In our case the detection of the various citric acid ethyl esters after methylation and additional silylation of the free hydroxyl group by gas chromatography in combination with mass spectrometric analysis, proved particularly suitable. The method used by us will be described below.

We tested freshly pressed juices of lemons, oranges, sour cherries, currents, and pineapples which had been liberated immediately of the fruit pulp by centrifuging.

Experimental part

1. Gas chromatography

Ethyl esters of citric acid When using a fractometer Perkin Elmner F 7. only citric acid triethyl ester appears in the gas chromatogram under the following conditions: R-column 1m, over temperature 170 degC, carrier gas: helium, velocity of flow: 150 ml/min, and current division 1:10 after the column, using a flame-ionization detector and charging 20 mu g of an esterification product of 1 mole citric acid and 2 moles ethanol, while citric acid-monoethyl esters and citric acid dimethyl esters are not indicated.

Mixture of the citric acid ethyl esters after methylation The esterification product yields after methylation with diazomethane under the same condition as indicated above 6 peaks of citric acid-trimethyl esters, symmetrical and asymmetrical citric acid-dimethyl-ethyl esters, symmetrical and asymmetrical citric acid-methyl-diethyl esters, and citric acid-triethyl esters.

Mixture of citric acid-ethyl esters after methylation and silylation For the reasons indicated in the chapter mass spectrometry, we used the esters obtained after methylation and silylation for the isolation and identification of the citric acid-ethyl esters from the juices. In the case of the intermediate isolation of the esters, we used the above indicated gas-chromographic conditions, reducing the retention time of the silylated esters, compared to those of the esters with free hydroxyl groups.

For recording the mass spectra of the pure substances from the mixture, we used a direct apparatus combination - gas chromatography-mass spectrometry. The gas-chromatographic fine separation was effected on a 100 m capillary column, and the effluate was introduced directly into the mass spectrometer.

Fig. 1 shows the mass spectrogram using a 100 m refined steel capillary with 0.5 mm inside diameter and polypropylene glycol 2000 as a stationary phase, oven temperature 170 degC, helium as a carrier gas with a velocity of flow of 3.5 ml/min and flame-ionization detector.

2. Mass spectrometry of the citric acid-ethyl ester derivatives

Since the mass spectra of the trialkyl esters of citric acid as hydroxyl compounds do not show a molecule peak, we tried from the beginning to effect the identification over the silyl ethers so that the mass spectra of the methylated and silylated citric acid-ethyl esters had to be used for the identification. Since the ethyl esters were not available in their symmetrical and asymmetrical forms as pure substances, we methylated and silylated the above mentioned ester mixture and obtained the mass spectra represented in fig. 2 to 7 according to the previously described method.

Fig. 1 Fractogram of the esterification mixture of 1 mole citric acid with 2 moles ethanol after methylation and silylation. Peaks 1 to 6: citric acid (1), symmetrical citric acid-ethyl ester (2),aasymmetrical citric acid ethyl ester (3), symmetrical citric acid-diethyl ester (4), symmetrical citric acid-diethyl ester (6), and citric acid-triethyl ester (6). Conditions: 100 m Gilay column, 0.5 mm inside diameter, polypropylene glycol 2000, oven temperature 170 degC, carrier gas helium FID.

The mass spectra of the silylated citric acid trialkyl esters have instead of the molecute peaks the M-15 peak typical of silyl ethers (6), with the fragments M/e 333, 319, 305 and 291. The spectra of the derivatives of the citric acid diethyl-esters and of the -monoethyl esters show differences in the peaks 275 and 261 resp., from which it can be seen clearly that we are dealing with 2 different esters; as it can also be concluded from the appearance of 2 derivatives from the fractogram (fig.1). The distinction between symmetrical and asymmetrical form, however, must be made in both cases first on the basis of the mass spectrum. We established the correlation therefore from the retention times in gas chromatography, where the molecule with the greater principal chain length should have the greater retention time. This view is supported by a study by Pinnow who found in esterification mixtures of citric acid with ethanol over the dissociation constants of the acid esters the asymmetrical monoethyl ester and the

symmetrical diethyl ester as the main portion (7).

Fig. 2-7 Mass spectra of the single components obtained from the esterification mixture of 1 mole citric acid and 2 moles ethanol after methylation and silylation. The measurement was made in an apparatus combination, gas chromatography-mass spectrometry. Conditions: 100 m Colay column, 0.5 mm inside diameter, polypropylene glycol 2000, oven temperature 170 degC, carrier gas helium, FID.

Fig. 2 Silylated trimethyl derivative of citric acid

Fig. 3 Silylated dimethyl derivative of the symmetrical monoethyl ester of citric acid

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Fig. 4 Silylated dimethyl derivative of the asymmetrical monoethyl ester of citric acid

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Fig. 5 Silylated methyl derivative of the asymmetrical diethyl ester of citric acid

---

F ig 6 Silylated methyl derivative of the symmetrical diethyl ester of citric acid

---

Fig. 7 Silylated citric acid-triethyl ester

The apparatus combination consisted of a Hitachi Perkin Elmer RMU-6 D mass - spectrometer, to the inlet of which was connected a specially developed gas-chromatograph with the above described 100 m Golay column.

3. Isolation of the ethyl esters of citric acid from fruits

25 kg fresh fruits (lemons, oranges, sour cherries, current and pineapples) were pressed in a fruit press or ketchen juicer and the juice obtained was liberated of the fruit pulp by centrifuging.

In order to obtain the neutral esters, the raw juices of the fruits were standardized with sodium hydrogen carbonate to a pH of 7.1 to 7.2, and subsequently extracted in a stirring apparatus with 4 to 6 liter dichloromethane.

The acid citric acid-dialkyl esters were obtained by extraction with

dichloromethane after acidification of the aqueous sodium hydrogen carbonate phase with 10% sulfuric acid to pH 2 to 3.

Attempts to obtain the monoalkyl esters of citric acid by extraction of the aqueous phase failed; added monoalkyl ester could not be shaken out after concentration of such an aqeuous phase. Distribution tests with the pure substances also showed that the monoalkyl esters can not be isolated this way. The diethyl esters are likewise still so hydrophilic that the bulk is found under certain circumstances only after concentration of the water phase.

4. Column chromatography of the ethyl esters of citric acid and of the methyl-ethyl-esters resp. of citric acid

Preliminary tests had shown that the carboxyl-containing esters of citric acid can not be readily eluted from kieselguhr. It was therefore found expedient to methylate the raw extracts already, and to effect the further isolation on trialkyl esters. The methylation was effected with diazomethane. The citric acid-trialkyl esters are washed down from siliga gel columns by a mixture of 40% hexane and 60% benzene.

5. Detection of citric acid triethyl ester in fruits

The dichloromethane extracts of the neutral substances obtained at pH 7.1 were evaporated to dryness and the evaporation residue was chromatographed over a column (diameter 10 mm) of 10 g silica gel (Merck, grain size 0.05 to 0.2 mm). The amount was taken up in hexane and eluted by hexane with increasing benzene portions. The citric acid triethyl ester issues at a benzene portion of about 60%.

For the identification, the fractions containing citric acid triethyl ester were combined, evaporated, and mixed in 0.7 ml pyridine with 0.2 ml hexamethyl-disilazane and 0.1 ml trimethyl silyl chloride. The mixture was sealed in an ampoule and heated for 50 minutes to 100 degC. After concentration to about 1/10 of the original volume, the reaction mixture was separated according to the above described gas-chromatographic separation method, and

the effluate was collected in ethyl acetate after the retention time determined on the pure substance for the mass-spectrometic identification. Citric acid-triethyl ester was found in the neutral dichloromethane-extracts of sour cherries and currants. Fig. 8 shows the mass spectrum of the silylated triethyl ester from sour cherries.

Fig. 8 Mass spectrum of citric acid-triethyl ester after silylation isolated from sour cherries.

Fig. 9 Mass spectrum of a mixture of symmetrical and asymmetrical citric acid-diethyl esters after methylation and silylation, isolated from lemons.

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6. Detection of the citric acid-diethyl ester-isomers from fruits

The dichloromethane extracts obtained after acidification of the fruit juices were evaporated, taken up in ether, and methylated by adding diazomethane in ether. Column chromatography and silylation were carried out in the same manner as described in chapter 5; for the gas chromotography and mass spectrometry applied the same conditions as indicated there. Fig. 8 shows a mixed spectrum of symmetrical and asymmetrical diethyl esters from the preparation of lemons after methylation and silylation.

Results

The results of the preparation of the five fruit juices are compiled in the following table. Measured by the rather high concentration which the citric acid has in the various fruits, the contents of citric acid esters are extremely low. The appearance of the citric acid triethyl ester and of the two isomeric forms of the citric acid diethyl ester prove that all carboxyl groups of citric acid are accessible to esterification in vivo. Though the monoethyl esters could not be detected with the isolation method, their intermediate appearance on the way to the higher esters can be considered as certain.

Table Detection of the ethyl esters of citric acid in various fruits

fruit	triethyl ester	symm.diethyl ester	asymm.diethyl ester
lemons	-	+	+
oranges	-	+	-
sour cherries	+	-	-
pineapples	-	-	-
red currants	+	-	-

References

Footnote

*) During the printing of this article we learned that Jansen, Palmer and Lundin have isolated symmetrical citric acid monoethyl ester from ripe green avocado pears. (8).

(Translated by Carl Demrick Associates,Inc/IE/t)

Figure 1

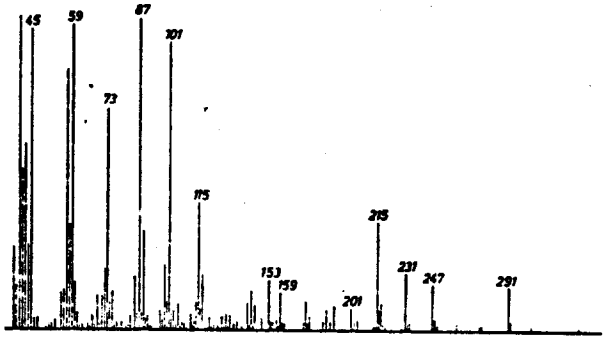

Figure 2

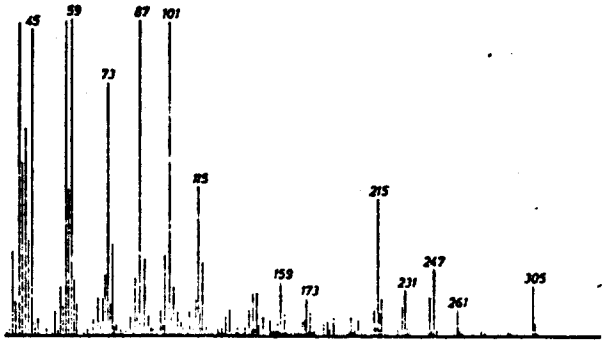

Figure 3

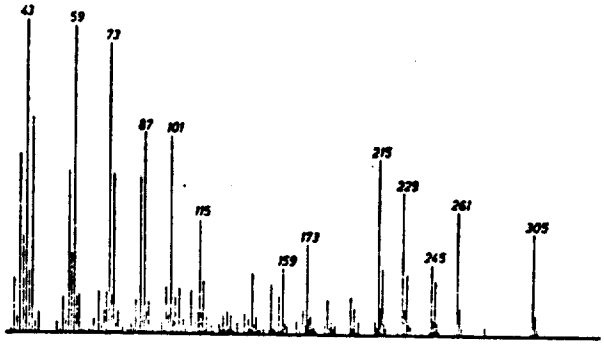

Figure 4

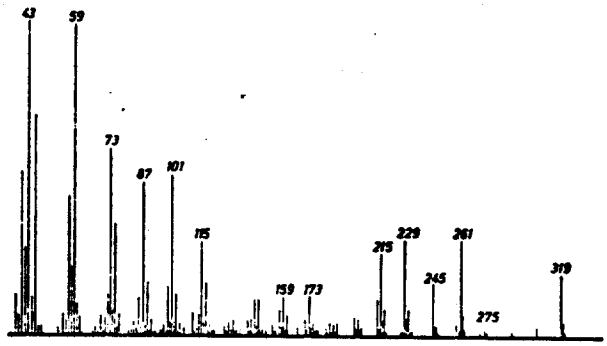

Figure 5

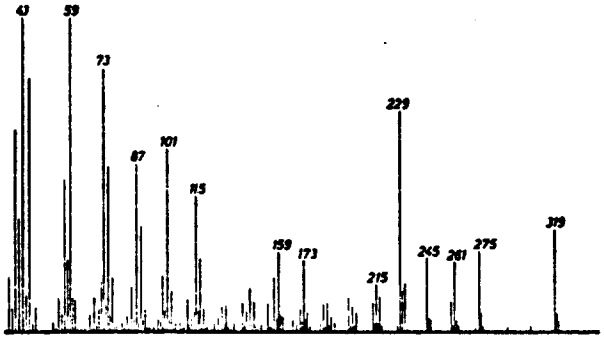

Figure 6

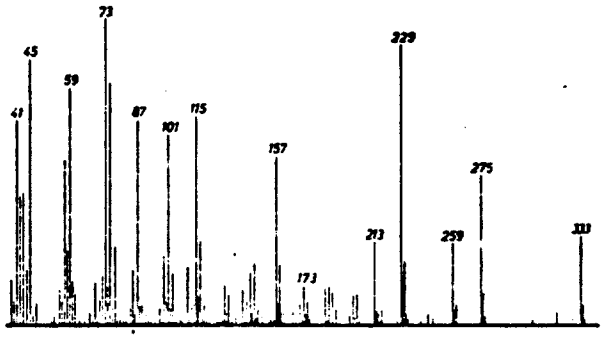

Figure 7

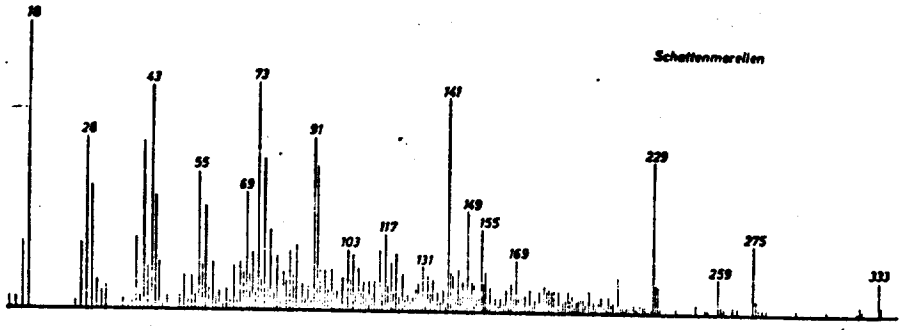

Figure 8

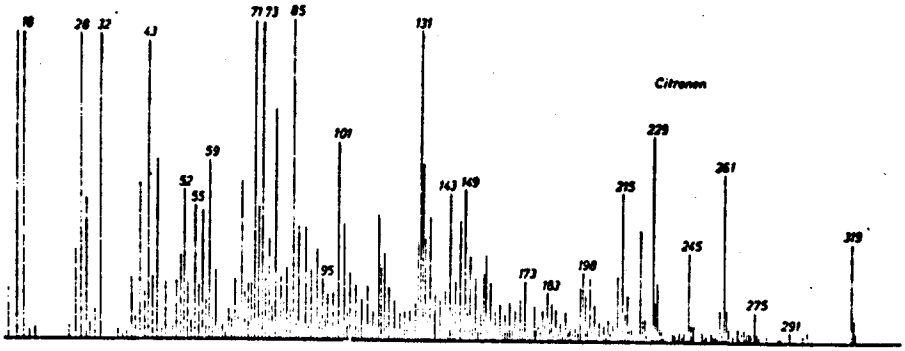

Figure 9

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Über das natürliche Vorkommen von Äthylestern der Citronensäure

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Mitteilung aus dem Zentrallaboratorium der Firma H. F. & Ph. F. Reemtsma, Hamburg

Eingegangen am 14. Juli 1967

Trotz der außerordentlichen Verbreitung der Citronensäure als Stoffwechselprodukt im Pflanzen- und Tierreich und ihrer auftretenden hohen Konzentrationen, die z. B. im Zitronensaft 5 bis 7% betragen, ist bisher über das Vorkommen der Ester der Citronensäure wenig bekannt geworden. Lediglich der Citronensäure-trimethylester und der symmetrische Citronensäure-dimethylester (Citronensäure-α,α'-dimethylester) wurden von BOSE und BHATTACHARYYA (1) in einer Arbeit aus dem Jahre 1956 in „*Ananas sativus*" nachgewiesen. PINNOW beschrieb 1915 die Isolierung eines Citronensäure-äthylesters ohne nähere Bezeichnung aus vergorenem Zitronensaft (2)*.

Es erschien daher interessant, dieses Gebiet unter Anwendung moderner Trenn- und Identifizierungsmethoden erneut aufzugreifen, zumal die Citronensäure und ihre Ester heute in der Lebensmitteltechnologie eine weit verbreitete Anwendung finden, z. B. als Kombinationspartner mit Antioxydantien (Synergisten) (3, 4), wobei ihre Wirkung in einer Inaktivierung von Metallionen besteht, und als Weichmacher (5).

Zum analytischen Nachweis von Polycarbonsäure-alkylestern ist in der Literatur bislang kein allgemein anwendbares Verfahren beschrieben worden. Während die neutralen Ester sich ohne weiteres gaschromatographisch isolieren lassen, gelingt die Gaschromatographie der Ester mit freien Carboxylgruppen nach vorangegangener Methylierung, also nach Überführung in neutrale Ester. In unserem Falle erwies sich der Nachweis der verschiedenen Citronensäure-äthylester, nach Methylierung und zusätzlicher Silylierung der freien Hydroxylgruppe, durch Gaschromatographie in

* Während der Drucklegung dieser Arbeit wurde uns bekannt, daß JANSEN, PALMER und LUNDIN aus reifen, grünen Avocadobirnen asymmetrischen Citronensäure-monoäthylester isoliert haben (8).

Kombination mit einer massenspektrometrischen Analyse als besonders geeignet. Das von uns angewandte Verfahren ist nachstehend beschrieben.

Zur Untersuchung gelangten frische Preßsäfte von Zitronen, Orangen, Schattenmorellen, Johannisbeeren und Ananas, die durch sofortiges Zentrifugieren vom Fruchtfleisch befreit wurden.

Experimenteller Teil

1. *Gaschromatographie*

Äthylester der Citronensäure: Bei Verwendung eines Fraktometers Perkin Elmer F 7 tritt unter den Bedingungen: R-Säule 1 m, Ofentemperatur 170° C, Trägergas Helium, Strömungsgeschwindigkeit 150 ml/min und Strömungsteilung 1 : 10 nach der Säule, bei Verwendung eines Flammen-Ionisations-Detektors und der Eingabe von 20 µg eines Veresterungsproduktes von 1 mol Citronensäure mit 2 mol Äthanol, lediglich der Citronensäure-triäthylester im Gaschromatogramm auf, während Citronensäure-monoäthylester und Citronensäure-diäthylester nicht angezeigt werden.

Gemisch der Citronensäure-äthylester nach Methylierung: Das Veresterungsprodukt gibt nach anschließender Methylierung mit Diazomethan, unter den gleichen Bedingungen wie oben angegeben, 6 Peaks von Citronensäure-trimethylester, symmetrischem und asymmetrischem Citronensäure-dimethyl-äthylester, symmetrischem und asymmetrischem Citronensäure-methyl-diäthylester und Citronensäure-triäthylester.

Gemisch der Citronensäure-äthylester nach Methylierung und Silylierung: Aus den im Abschnitt Massenspektrometrie angeführten Gründen wurden zur Isolierung und Identifizierung der Citronensäure-äthylester aus den Früchten die nach Methylierung und Silylierung erhaltenen Ester verwendet. Im Falle der Zwischenisolierung der Ester wurden die oben angegebenen gaschromatographischen Bedingungen angewandt, wobei die Retentionszeiten der silylierten Ester gegenüber denen der Ester mit freier Hydroxylgruppe verkürzt sind.

Für die Aufnahme der Massenspektren der Reinsubstanzen aus dem Gemisch wurde eine direkte Gerätekombination, Gaschromatographie — Massenspektrometrie, verwendet. Die gaschromatographische Feintrennung wurde auf einer 100 m-Kapillarsäule vorgenommen und das Effluat direkt in das Massenspektrometer eingeleitet.

Abb. 1 zeigt das Gaschromatogramm bei Verwendung einer 100 m-Edelstahlkapillare mit 0,5 mm Innendurchmesser und Polypropylenglykol 2000 als stationärer Phase, Ofentemperatur 170° C, Helium als Trägergas mit einer Strömungsgeschwindigkeit von 3,5 ml/min und Flammen-Ionisations-Detektor.

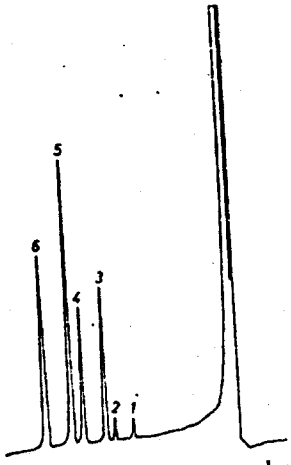


Abb. 1. *Fraktogramm des Veresterungsgemisches von 1 mol Citronensäure mit 2 mol Äthanol nach Methylierung und Silylierung.* Peak 1 bis 6: Citronensäure (1), symmetrischer Citronensäure-äthylester (2), asymmetrischer Citronensäure-äthylester (3), asymmetrischer Citronensäure-diäthylester (4), symmetrischer Citronensäure-diäthylester (5) und Citronensäure-triäthylester (6). Bedingungen: 100 m-Golaysäule, 0,5 mm Innendurchmesser, Polypropylenglykol 2000, Ofentemperatur 170° C, Trägergas Helium, FID

2. *Massenspektrometrie der Citronensäure-äthylester-Derivate*

Da die Massenspektren der Trialkylester der Citronensäure als Hydroxylverbindungen keinen Molekülpeak aufweisen, wurde von vornherein angestrebt, die Identifizierung über die Silyläther vorzunehmen, so daß zur Identifizierung die Massenspektren der methylierten und silylierten Citronensäure-äthylester herangezogen werden mußten. Da uns die Äthylester in ihren symmetrischen und asymmetrischen Formen als Reinsubstanzen nicht zugänglich waren, haben wir das angeführte Estergemisch methyliert, silyliert und nach dem vorher dargestellten Verfahren die in Abb. 2 bis 7 angegebenen Massenspektren erhalten.

Die Massenspektren der silylierten Citronensäure-trialkylester weisen anstelle des Molekülpeaks den für Silyläther typischen M-15 Peak (6) auf, wobei die Fragmente M e 333, 319, 305 und 291 auftreten. Die Spektren der Derivate der Citronensäure-diäthylester und der -monoäthylester weisen Unterschiede in den Peaks 275 bzw. 261 auf, aus denen klar erkennbar ist, daß jeweils 2 verschiedene Ester vorliegen; wie auch aus dem Auftreten von 2 Derivaten aus dem Fraktogramm (Abb. 1) geschlossen werden kann. Die Unterscheidung zwischen symmetrischer

und asymmetrischer Form ist jedoch in beiden Fällen anhand des Massenspektrums vorerst nicht zu treffen. Wir haben die Zuordnung deshalb aus den Retentionszeiten in der Gaschromatographie getroffen, wobei das Molekül mit der größeren Hauptkettenlänge die größere Retentionszeit aufweisen sollte. Die Auffassung wird gestützt durch eine Arbeit von Pinnow, der in Veresterungsgemischen von Citronensäure mit Äthanol, über die Dissoziationskonstanten der sauren Ester, als Hauptanteil den asymmetrischen Monoäthylester und den symmetrischen Diäthylester fand[7].

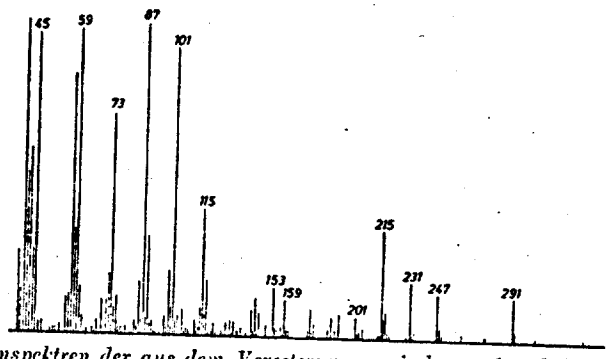


Abb. 2—7. *Massenspektren der aus dem Veresterungsgemisch von 1 mol Citronensäure und 2 mol Äthanol nach Methylierung und Silylierung gewonnenen Einzelkomponenten.* Die Messung erfolgte in einer Gerätekombination, Gaschromatographie — Massenspektrometrie. Bedingungen: 100 m Golaysäule, 0,5 mm Innendurchmesser, Polypropylenglykol 2000, Ofentemperatur 170° C, Trägergas Helium, FID

Abb. 2. *Silyliertes Trimethylderivat der Citronensäure*

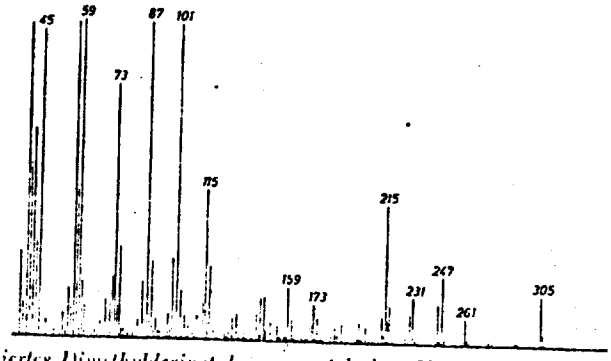


Abb. 3. *Silyliertes Dimethylderivat des symmetrischen Monoäthylesters der Citronensäure*

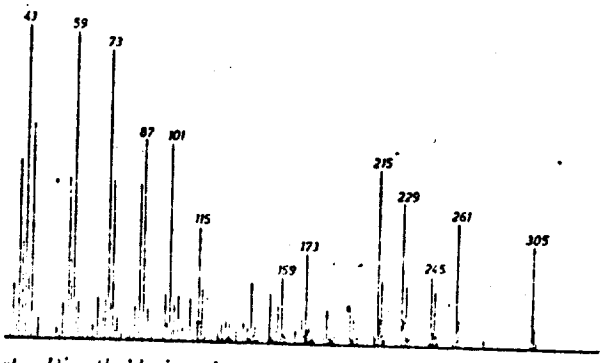


Abb. 4. *Silyliertes Dimethylderivat des asymmetrischen Monoäthylesters der Citronensäure*

Die Gerätekombination bestand aus einem Hitachi Perkin Elmer RMU-6 D Massenspektrometer, an dessen Einlaßteil ein speziell entwickelter Gaschromatograph mit der beschriebenen [illegible] m-Golay-Säule angeschlossen war.

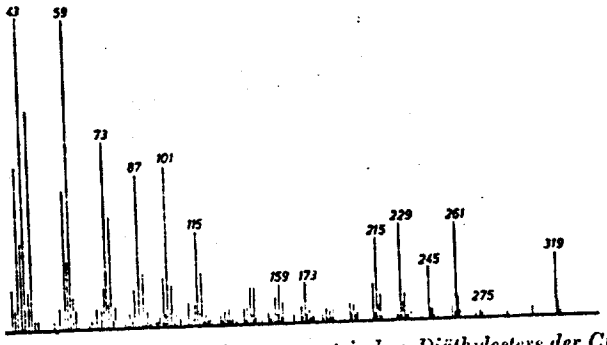


Abb. 5. *Silyliertes Methylderivat des asymmetrischen Diäthylesters der Citronensäure*

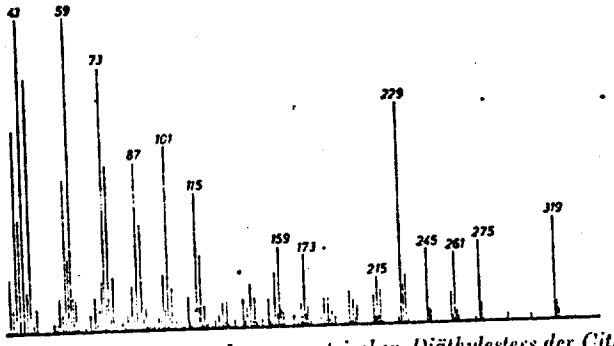


Abb. 6. *Silyliertes Methylderivat des symmetrischen Diäthylesters der Citronensäure*

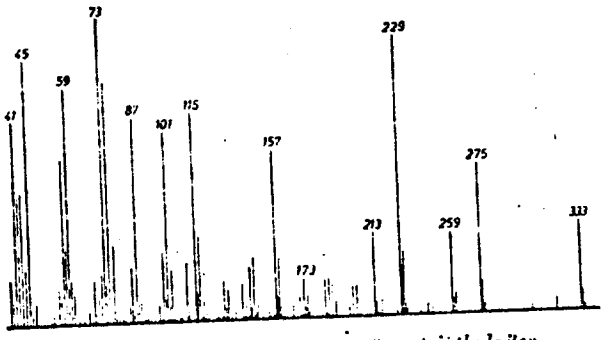


Abb. 7. *Silylierter Citronensäure-triäthylester*

3. *Isolierung der Äthylester der Citronensäure aus Früchten*

25 kg frischer Früchte (Zitronen, Orangen, Schattenmorellen, Johannisbeeren und Ananas) wurden in einer Fruchtpresse bzw. einem Küchenentsafter ausgepreßt und der gewonnene Saft durch Zentrifugieren vom Fruchtfleisch befreit.

Zur Gewinnung der neutralen Ester wurden die Rohsäfte der Früchte mit Natriumhydrogencarbonat auf pH-Wert 7,1 bis 7,2 eingestellt und anschließend mit 4 bis 6 l Dichlormethan in einer Kohrapparatur extrahiert.

Die sauren Citronensäure-dialkylester wurden, nach Ansäuern der wäßrigen Natriumhydrogencarbonat-Phase mit 10%iger Schwefelsäure auf pH 2 bis 3, durch anschließende Extraktion mit Dichlormethan gewonnen.

Versuche, die Monoalkylester der Citronensäure durch Extraktion der wäßrigen Phase zu gewinnen, schlugen fehl; zugesetzter Monoalkylester konnte auch nach Einengen einer solchen wäßrigen Phase nicht ausgeschüttelt werden. Auch Verteilungsversuche mit den Reinsubstanzen zeigten, daß die Monoalkylester auf diese Weise nicht isoliert werden können. Auch die Diäthylester sind noch so hydrophil, daß die Hauptmenge unter Umständen erst nach Einengen der Wasserphase aufgefunden wird.

4. Säulenchromatographie der Äthylester der Citronensäure bzw. der Methyl-äthylester der Citronensäure

Vorversuche zeigten, daß die carboxylhaltigen Ester der Citronensäure von Kieselgel nicht ohne weiteres eluierbar sind. Es erwies sich daher als zweckmäßig, bereits die Rohextrakte zu methylieren und die weitere Isolierung an Trialkylestern durchzuführen. Die Methylierung wurde mit Diazomethan durchgeführt. Die Citronensäure-trialkylester werden von Kieselgel-Säulen durch ein Gemisch von 40% Hexan und 60% Benzol heruntergewaschen.

5. Nachweis des Citronensäure-triäthylesters in Früchten

Die bei pH-Wert 7,1 erhaltenen Dichlormethan-Extrakte der neutralen Stoffe wurden zur Trockne eingedampft und der Abdampfrückstand über eine Säule (Durchmesser 10 mm) von 10 g Kieselgel (Merck, Korngröße 0,05 bis 0,2 mm) chromatographiert. Die Menge wurde in Hexan aufgenommen und durch Hexan mit steigenden Benzolanteilen eluiert. Der Citronensäure-triäthylester tritt bei einem Benzolanteil von etwa 60% aus.

Zur Identifizierung wurden die Citronensäure-triäthylester enthaltenden Fraktionen vereinigt, eingedampft und in 0,7 ml Pyridin mit 0,2 ml Hexamethyldisilazan und 0,1 ml Trimethylsilylchlorid versetzt. Das Gemisch wurde in eine Ampulle eingeschmolzen und 50 min auf 100° C

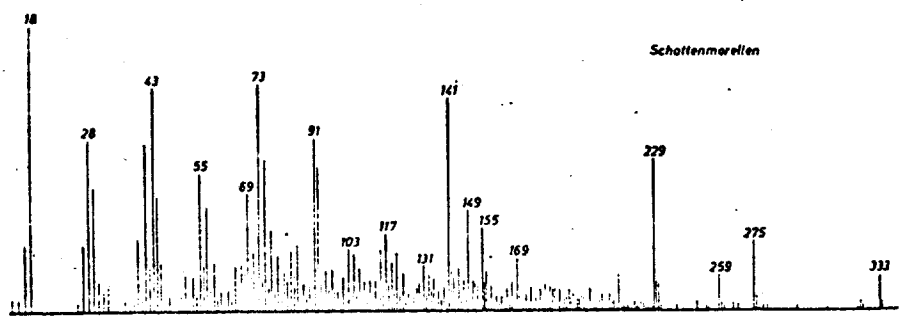


Abb. 8. *Massenspektrum von Citronensäure-triäthylester nach Silylierung, aus Schattenmorellen isoliert*

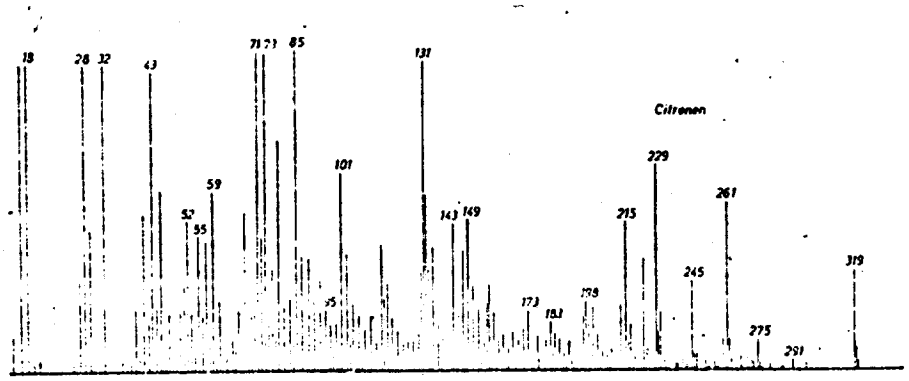


Abb. 9. *Massenspektrum einer Mischung von symmetrischem und asymmetrischem Citronensäure-diäthylester nach Methylierung und Silylierung, aus Zitronen isoliert*

...en Natriumhydrogen-
...ßende Extraktion mit

...der wäßrigen Phase zu
...Einengen einer solchen
...mit den Reinsubstanzen
...nnen. Auch die Diäthyl-
...nach Einengen der Was-

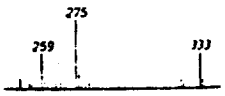
...r Methyl-äthylester

...äure von Kieselgel nicht
...reits die Rohextrakte zu
... Die Methylierung wurde
...en von Kieselgel-Säulen
...n.

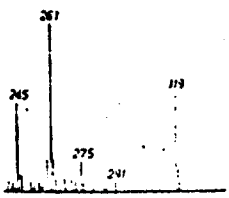
...hten

...ralen Stoffe wurden zur
...urchmesser 10 mm) von
...t. Die Menge wurde in
...iert. Der Citronensäure-

...den Fraktionen vereinigt,
...d 0,1 ml Trimethylsilyl-
... und 50 min auf 100° C



...ung, aus Schattenmorellen



...ametrischem Citronensäure
...onen isoliert

erhitzt. Nach dem Einengen auf ca. $^1/_{10}$ des Ausgangsvolumens wurde das Reaktionsgemisch nach dem eingangs dargestellten gaschromatographischen Trennverfahren aufgetrennt und das Effluat nach der an der Reinsubstanz ermittelten Retentionszeit in Äthylacetat zur massenspektrometrischen Identifizierung aufgefangen. Citronensäure-triäthylester wurde in den neutral erhaltenen Dichlormethanextrakten von Schattenmorellen und Johannisbeeren nachgewiesen. Abb. 8 gibt das Massenspektrum des silylierten Triäthylesters aus Schattenmorellen wieder.

6. Nachweis der Citronensäure-diäthylester-Isomeren aus Früchten

Die nach Ansäuern der Fruchtsäfte erhaltenen Dichlormethan-Extrakte wurden eingedampft, in Äther aufgenommen und durch Zusatz von Diazomethan in Äther methyliert. Säulenchromatographie und Silylierung wurden in gleicher Weise durchgeführt wie im Abschnitt 5 beschrieben; auch für die Gaschromatographie und Massenspektrometrie galten die gleichen Bedingungen wie dort angegeben. Abb. 9 zeigt ein Mischspektrum von symmetrischem und asymmetrischem Diäthylester aus der Zitronenaufarbeitung nach Methylierung und Silylierung.

Ergebnisse

Die Ergebnisse der Aufarbeitung der fünf Fruchtsäfte sind in der Tabelle zusammengestellt. Gemessen an der recht hohen Konzentration, die die Citronensäure in den einzelnen Früchten aufweist, sind die Gehalte an Citronensäure-estern außerordentlich gering. Das Auftreten des Citronensäure-triäthylesters und der beiden isomeren Formen des Citronensäure-diäthylesters beweisen, daß alle Carboxylgruppen der Citronensäure der Veresterung in vivo zugänglich sind. Wenn auch bei der Isolierungsmethodik die Monoäthylester nicht erfaßt werden konnten, so ist doch ihr intermediäres Auftreten auf dem Wege zu den höheren Estern als sicher anzusehen.

Tabelle. *Nachweis der Äthylester der Citronensäure in verschiedenen Früchten*

Frucht	Triäthylester	symm. Diäthylester	asymm. Diäthylester
Zitronen	—	+	+
Orangen	—	+	—
Schattenmorellen	+	—	—
Ananas	—	—	—
Rote Johannisbeeren	+	—	—

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Received December 28, 1966

J. BIOMED. MATER. RES. VOL. 1, PP. 197–211 (1967)

Growth Changes in Mammalian Cell Cultures by Plastic Additives (Effect of Triethyl Citrate)*

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Summary

Inhibition of cultured mammalian cell growth was used to examine the biological activity of triethyl citrate, a common plasticizer for poly(vinyl chloride). Cell populations were determined by a simple extraction procedure and measurement of nucleic acid content (total purines and pyrimidines) by its absorbance at 268 mμ. Triethyl citrate demonstrated strikingly different dose–effect characteristics from its theoretical hydrolysis products (ethanol and citric acid). The inhibitory effects of sodium citrate were reversed by calcium chloride while the action of triethyl citrate underwent little or no change under similar conditions. Triethyl citrate did not appear to produce resistant cell strains in culture but did seem to be degraded with time. Inhibitory action of triethyl citrate was shown to be independent of inoculum size within the ranges utilized in the study. The procedures described appear to have a number of advantages for the biological or toxicological evaluation of ingredients which might be added to a plastic formulation or to contaminates which might enter the plastic during manufacture of the item or device.

I. INTRODUCTION

With the ever-increasing use of man-made materials in medicine, dentistry, pharmacy, and the biomedical and bioengineering fields, greater caution and concern must be exerted to prevent the introduction of those materials which may have potential harm to biological systems. In recent years reports have appeared which have focused

* Presented at the Scientific Session, A.Ph.A. Meeting, Dallas, Texas, April, 1966. This investigation was supported by Grant CA-06120 from the National Cancer Institute, U.S. Public Health Service, Bethesda, Maryland.

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attention on the possible toxicological or untoward effects various types of devices might have if proper evaluation is not made of the material.[1–4] Our own laboratories have for a number of years conducted routine biological testing of materials which might enter a medical or para-medical application.[5] One of our routine test procedures utilizes mammalian cells as test organisms for detecting the presence of toxic agents in a material.[6,7] This method has proven successful as a rapid screening test to ferret out undesirable materials from a biological standpoint.

Since most plastic materials will generally have one or more agents added to the formula or to the final device or item, it was decided to further utilize mammalian cell cultures in evaluating the biological properties of additives which might become a part of a finished plastic item. A method was required which would not depend upon cellular death as an endpoint, be sensitive to microquantities of test materials, have high reproducibility, and which would lend itself to quantitation. This report describes methodology for determining growth inhibitory properties of additives utilizing strain L mouse fibroblasts. Triethyl citrate was chosen as the additive (plasticizer) since it is relatively nontoxic by oral administration but has been found to elicit a higher order of activity when parenterally administered.[8,9]

II. EXPERIMENTAL

A. Supplies

Triethyl citrate (99% pure), Chas. Pfizer & Co., Brooklyn, New York.
Citric acid, anhydrous, A.C.S. Fisher Scientific Co., Fair Lawn, New Jersey.
Sodium citrate, dihydrate, A.R., J. T. Baker Chemical Co., Phillipsburg, New Jersey.
Calcium chloride, A.C.S., J. T. Baker Chemical Co., Phillipsburg, New Jersey.
Ethanol, U.S.P., U.S. Industrial Chemical Co., New Orleans, Louisiana.
Phosphate Buffered Salt (Dulbecco) Solution:
- 8.0 g sodium chloride, A.C.S., J. T. Baker Chemical Co., Phillipsburg, New Jersey.
- 0.2 g potassium chloride, A.C.S., J. T. Baker Chemical Co., Phillipsburg, New Jersey.
- 1.15 g sodium phosphate, dibasic, A.C.S., J. T. Baker Chemical Co., Phillipsburg, New Jersey.
- 0.2 g potassium phosphate, monobasic, A.C.S., J. T. Baker Chemical Co., Phillipsburg, New Jersey.
- Sufficient deionized water to prepare 1000 ml.

Potassium Penicillin G, Chas. Pfizer & Co., Brooklyn, New York.
Streptomycin sulfate, Chas. Pfizer & Co., Brooklyn, New York.
Calf Serum, Microbiological Associates, Bethesda, Maryland.
Trypsin, 2.5% in Isotonic Saline Solution, Microbiological Associates, Bethesda, Maryland.
Basal Medium (Eagle) with Earle's Balanced Salt Solution, Microbiological Associates, Bethesda, Maryland.

B. Equipment

Culture tubes, 18 × 150 mm, with white rubber lined screw caps, Pyrex T136-41, Corning Glass Works, Corning, New York.
Magnetic stirrer and Teflon-coated bars, Precision Scientific Company, Chicago, Illinois.
Cornwall semiautomatic syringe pipet, 2.0 ml, Becton, Dickinson and Company, Rutherford, New Jersey.
Centrifuge, Model UV, International Equipment Company, Needham Heights, Massachusetts.
Blake culture bottles, 32 oz, T. C. Wheaton, Millville, New Jersey.
Incubator, Precision-Thelco J1510, Chicago, Illinois.
Spectrophotometer, Beckman DB, Fullerton, California.

C. Cultured Cell Strain

Strain L-929 [(Earle) Mouse Fibroblast Cells, carried in culture by the Drug-Plastic Research & Toxicology Laboratories; original inoculum obtained by courtesy of Dr. Royce Z. Lockart, Microbiology Department, The University of Texas].

D. Cell Culture Methods

Strain L mouse fibroblasts were maintained as monolayer cultures in Blake culture bottles. The medium used throughout the study consisted of Eagle's basal medium supplemented with 10% calf serum, containing 100 units of penicillin and 50 mcg of streptomycin per ml.[10] Cells were harvested with the aid of trypsin and suspended in fresh medium. After mixing over a magnetic stirrer for 10 min, cell populations were estimated by haemocytometer counts and further dilutions made as required for individual experiments.

A 2.0-ml semiautomatic Cornwall pipet was employed to withdraw and deliver a 1.5 ml aliquot containing 0.3 million cells into each culture tube. In growth inhibition studies each test culture received an added 1.0 ml dose of test compound (containing a graded concentration of test material dissolved in medium). Control cultures received an additional 1.0 ml of the medium. When two test

compounds were added to individual cultures, 0.5 ml quantities of each were utilized.

Baseline or time-zero population values were established by immediate determination of cell population in 7 to 10 control cultures by a method to be described in the next section. Screw caps of the remaining control and test cultures were loosely secured and the cultures incubated statically for designated periods of time.* Periodically, or as specified in individual experiments, five replicates of test and/or control cultures were removed from the incubator and their cell populations determined. Seven to 10 cultures were utilized as final controls in growth inhibition studies. Cell populations were determined to provide total (or 100%) growth data for cultures without the presence of any test compound.

Preliminary experiments established that the culture tubes could be incubated in either horizontal or vertical positions. For convenience cell cultures were subsequently incubated vertically. Other experiments demonstrated that with the L cells, under the conditions described, the logarithmic growth phase occurred between 40 and 90 hr. Growth inhibition studies were terminated after 72 hr of growth, except if otherwise stated.

E. Determination of Cell Population

Sulfuric acid extracts of strain L cultures exhibit a characteristic absorption maximum at 265–270 mμ. This appears to be due to purines and pyrimidines, of which DNA and RNA represent about 75–80% of the total.[11] Absorbance values have been demonstrated to be related linearly to number of strain L cells as well as to other types of cells.[11,12]

In this report cell population determination methods were adapted from procedures originally introduced by McIntire and Smith and modified by Hori et al.[11,12] The method follows the procedure described below.

Cultures to be analyzed are washed twice with 5.0-ml quantities of phosphate-buffered salt solution (PBS). If cells are not firmly attached to culture tubes, centrifugation at 2000 rpm for 15 min is utilized prior to and between washings. If firmly attached, the PBS is pipetted slowly down the slanted walls of the culture tubes and the centrifugation step eliminated. After the second washing, 2.5 ml of 0.1N sodium hydroxide solution is introduced to each culture tube to detach

* At 35°C in a humidified 5% CO_2–air atmosphere.

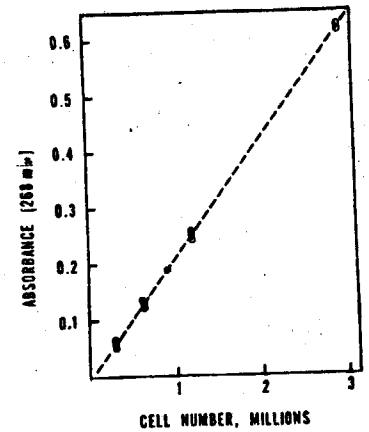

Fig. 1. Relationship between cell number and absorbance.

cells from the glass. The tubes are shaken and 2.5 ml of 0.3N sulfuric acid containing 20% (w/v) sodium chloride is added. The cultures are then mixed thoroughly and heated in boiling water for 15 min. Finally, the cultures are cooled below 20°C, mixed, and centrifuged at 2000 rpm for 15 min. The supernatant liquid is then examined spectrophotometrically at a wavelength of 268 mμ.

Absorbance values for individual cultures were shown to be a linear function of cell number in a range of 0.3 to 3.0 million cells, as may be seen in Figure 1.

F. Effect of Triethyl Citrate Concentration on Growth of Cell Cultures

A series of cultures containing 0, 3, 6, 9, and 12 × 10^{-3} moles/l of triethyl citrate were prepared and incubated as previously described. Over periods of time extending to approximately 72 or 80 hr, tubes were withdrawn and the cell populations determined. Figure 2 shows a plot of absorbance versus time for the various concentrations of triethyl citrate and clearly demonstrates the reduction of growth in cultures containing increasing concentrations of the ester.

In additional experiments utilizing triethyl citrate within the above concentration range, only initial (0 hr) and final (72 hr) cell populations were determined. The data were calculated as per cent growth using the simple expression:

$$\% \text{ growth} = \frac{A - B}{A_0 - B} \times 100$$

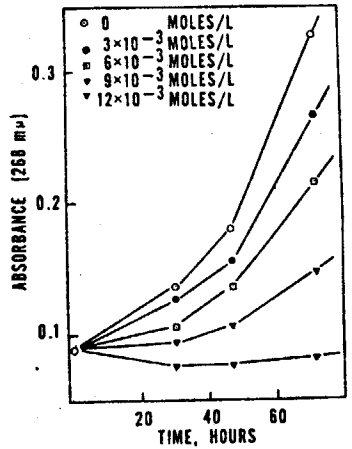

Fig. 2. Growth curves for strain L cells in the presence of graded concentrations of triethyl citrate.

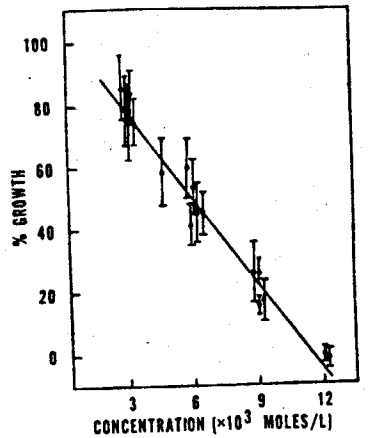

Fig. 3. Dose–response curve for triethyl citrate.

where A is the final mean absorbance value for a given concentration of test compound, A_0 is the final mean absorbance value for controls (zero concentration of test compound), and B is the initial mean absorbance value of cultures at the start of the incubation period. A dose–response curve for triethyl citrate is shown in Figure 3 and was compiled from five individual experiments. The method of

least squares was used to fix the line. From this type of curve it is also possible to evaluate (through least-square equations) the ID_{50} or the inhibitory dose 50 value (the concentration of individual agent inhibiting growth to one-half that of controls).

G. Effect of Sodium Citrate Concentration on Growth of Cell Cultures

In order to determine the effects of the citrate ion (one of the hydrolysis products of triethyl citrate) on the growth of cell cultures, sodium citrate was examined. Growth inhibition experiments were conducted using concentrations of the test agent ranging between 2 and 12×10^{-3} moles/l. Incubation of the organism was continued for 72 hr at which time cell populations were determined. The resultant dose–response curve is shown in Figure 4, where it may be observed that sodium citrate showed little inhibitory effect on growth at concentrations up to 5×10^{-3} moles/l. Dramatic inhibitory effects did, however, appear within the concentration range of 6.0 to 9.0×10^{-3} moles/l, wherein inhibition approached a maximum (cellular death). Figure 4 shows two dose–response curves, one of which is attributed to the sodium citrate. The second curve (sodium citrate plus calcium chloride) will be alluded to in another section. The plots shown in Figure 4 contain the actual values obtained from the experiments.

Figure 5 compares the dose–response curves for sodium citrate and triethyl citrate but the actual values have not been designated on

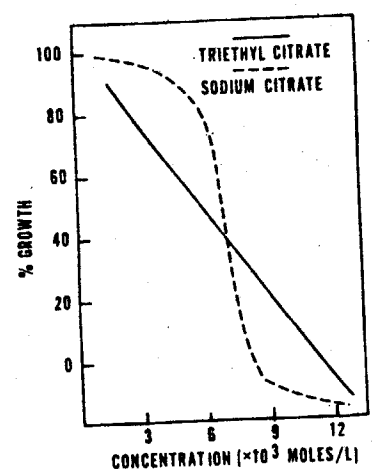

Fig. 4. Comparison of dose–response curves of triethyl citrate and sodium citrate.

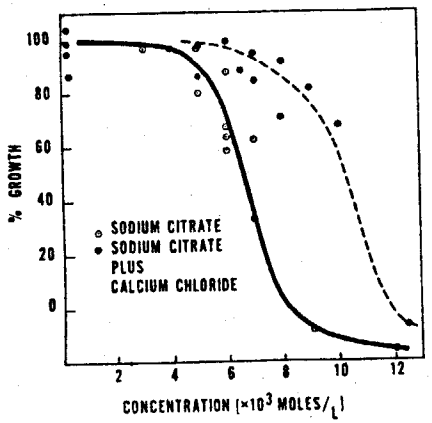

Fig. 5. Dose–responses for sodium citrate alone and with excess calcium chloride

the plots in order to avoid confusion. It will be noted that the ID_{50} values are similar (i.e., $6.97 \pm 0.33 \times 10^{-3}$ moles/l for sodium citrate and $5.96 \pm 0.20 \times 10^{-3}$ moles/l for triethyl citrate. An analysis of the dose-response curves (Fig. 5), however, reveals marked differences. The sodium citrate curve is relatively steep as compared to the triethyl citrate curve. At concentrations less than 6.0×10^{-3} moles/l, triethyl citrate is shown to be considerably more reactive than sodium citrate. Beyond 8.0×10^{-3} moles/l, the ester exhibits far less inhibitory action than does sodium citrate. In the latter range sodium citrate has approached maximum activity while the effects of triethyl citrate continue to be a linear function of concentration.

If it is postulated that triethyl citrate is hydrolyzed in culture and that its action may be due to the released citrate ion, it should be reasonable to expect sodium citrate to be more reactive at all concentrations. That is, one would expect the sodium citrate dose–response curve to fall below that of triethyl citrate throughout the inhibitory concentration range. Since this is not the case, it appears that the inhibitory action of triethyl citrate is not due to citrate ion toxicity.

H. Effect of Ethanol Concentration on Growth of Cell Cultures

Results from the previous experiments revealed differences in the inhibitory effects of triethyl citrate and one of its hydrolysis products, citric acid (as ion). The contributory effect of the other hydrolysis

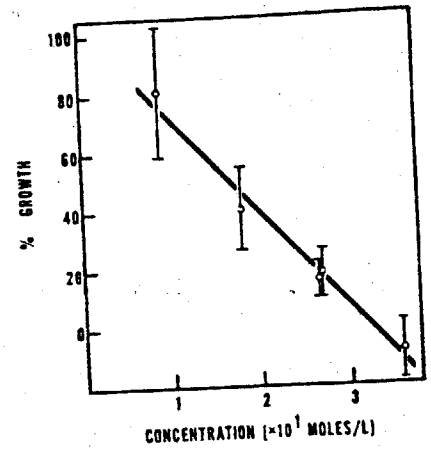

Fig. 6. Dose–response curve for ethanol.

product, ethanol, should now be considered. If complete hydrolysis of triethyl citrate is proposed, 3 moles of ethanol (and 1 mole of citric acid) would result from each mole of the ester.

To determine the effect of ethanol, two growth inhibition experiments were conducted similar to the ones previously described. Cultures containing up to 3.6×10^{-1} moles/l of ethanol were incubated for 72 hr. Calculations based on the initial and final cell populations resulted in a dose–response curve as shown in Figure 6. An analysis of this curve dramatizes the negligible inhibitory effect of ethanol. In order to reduce the growth of cell cultures by one-half, ethanol must be present in a concentration 20 times greater than the ID_{50} value of the intact ester. Toxicity effects to cells cannot thus be related to the hydrolysis products of the ester, even if one assumes that the ester is completely hydrolyzed.

I. Effect of Calcium Chloride on Growth Inhibitory Action of Sodium Citrate on Cell Cultures

Calcium chloride has been observed to reverse the action of sodium citrate in a number of biological systems.[13–17] A series of three growth inhibition experiments was conducted to determine whether calcium chloride could significantly alter the growth inhibitory effect of sodium citrate in the cell culture system employed in this investigation. Cultures containing 3.6×10^{-3} moles/l of calcium chloride

and concentrations ranging from 0 to 12 × 10^{-3} moles/l of sodium citrate were prepared. The additional calcium chloride doubled the usual concentration (1.8 × 10^{-3} moles/l) of this compound in the medium. Controls included cultures which contained neither additional calcium chloride nor sodium citrate. Additional medium was added to the control cells, resulting in a final volume of 2.5 ml in each tube. Initial and final (after 72 hr of incubation) cell populations were utilized in calculating per cent growth values. The dose–response curve has been included in Figure 4 to show the effect of calcium chloride in reducing the toxicity of sodium citrate. Subsequent calculations showed the ID_{50} value for sodium citrate to be increased by the presence of calcium chloride from 6.97 × 10^{-3} moles/l to more than 10.0 × 10^{-3} moles/l. As with other biological systems, calcium chloride did reverse the sodium citrate effect on the L cells.

J. Effect of Calcium Chloride on Growth Inhibitory Action of Triethyl Citrate on Cell Cultures

It has been demonstrated that the hydrolysis products of triethyl citrate are not the agents inhibiting the growth of L cells when the ester is added to the culture. To further confirm this observation another experiment was designed to note if calcium chloride might

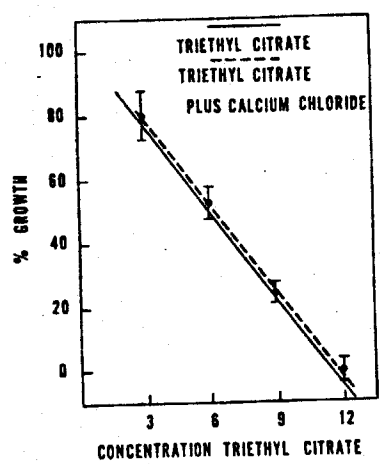

Fig. 7. Dose–response curves for triethyl citrate alone and with excess calcium chloride.

reverse the action of the ester (or the possible citrate released to the medium) as demonstrated in the experiment reported in the previous section when sodium citrate was investigated. Cultures were prepared, as in the foregoing experiment, with 3.6 × 10^{-3} moles/l of calcium chloride, but containing triethyl citrate in concentrations ranging from 0 to 12 × 10^{-3} moles/l. Controls included cultures which contained neither calcium chloride nor triethyl citrate. The cultures were incubated and their cell populations determined as previously related. Figure 7 contains dose–response plots for the triethyl citrate plus calcium chloride experiment and for the triethyl citrate (alone) experiment. Calcium chloride is shown to have little or no effect on the growth inhibitory properties of triethyl citrate. The ID_{50} values of 5.96 ± 0.20 × 10^{-3} moles/l for triethyl citrate and 6.28 ± 0.22 × 10^{-3} moles/l for triethyl citrate with additional calcium chloride do not differ significantly ($P = 0.05$).

K. Effect of Triethyl Citrate Concentration on Growth of Cell Cultures During Logarithmic Growth

To gain information as to the early effects of triethyl citrate, an experiment was conducted to test the activity of the ester toward rapidly growing cultures. Following the methods of Brown and Garrett, 2.0-ml cultures were allowed to attain logarithmic growth

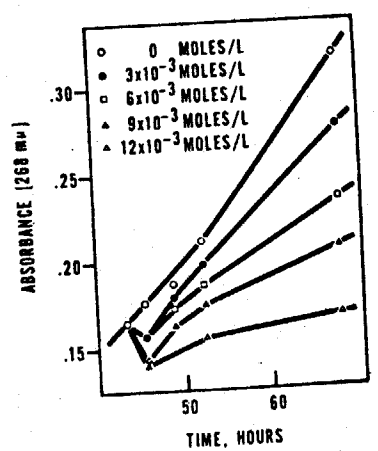

Fig. 8. Growth curves for strain L cells in the presence of graded concentrations of triethyl citrate added during logarithmic growth.

(after 43.5 hr of incubation) prior to the addition of graded concentrations of triethyl citrate in 0.5-ml aliquots.[18] Controls received 0.5 ml of medium. Cultures then contained 0, 3, 6, 9, and 12 $\times 10^{-3}$ moles/l of triethyl citrate. Cell populations were determined at intervals in replicates of 3 to 4 cultures at each dose level. The results were plotted as absorbance versus time as demonstrated in Figure 8. The curves reveal an almost immediate reduction in absorbance (cell population) following the administration of the triethyl citrate. Higher concentrations of the ester are associated with more rapid and steeper descents, which apparently reach a minimum within 4 hr.

The results depicted in Figure 8 may be interpreted as being due to one or more factors, the most important ones being that (*a*) the ester was consumed or degraded with time, (*b*) cells developed resistance with each succeeding generation, and/or (*c*) triethyl citrate-resistant strains of cells were present in the original culture.[18,19] To attempt to ascertain which of the previous suggestions are valid, the following experiment was conducted.

Medium in two of three culture bottles containing sparse monolayers of normal cells were replaced with freshly prepared medium containing 9 $\times 10^{-3}$ moles/l of triethyl citrate. Medium in the third bottle was replaced with fresh medium only. After incubation for 5 hr, fluids from all three bottles were removed and cell layers were washed twice with medium. Freshly prepared medium was added to the bottles. After incubating for 40 hr, cells were harvested and the two cultures which were treated with triethyl citrate were combined. A growth inhibition experiment was conducted in which sets of ester-treated and untreated (controls) cultures each received graded concentrations of triethyl citrate. The final concentrations of triethyl citrate in each set were 0, 3.29, 6.59, and 9.88 $\times 10^{-3}$ moles/l. Initial and final cell populations were determined and per cent growth values calculated. These values are listed in Table I. The ID_{50} value of triethyl citrate for the pretreated cultures did not differ significantly from the ID_{50} value for the untreated controls (Table I). It thus appears that cell strains resistant to the effects of triethyl citrate neither were originally present nor developed in cell cultures. The remaining possibility, therefore, points to the consumption or degradation of the ester as the best explanation for the growth pattern (Fig. 8) following administration of triethyl

citrate. This conclusion is further substantiated by the short (4 hr) period prior to recovery and the reported enzymatic hydrolysis of triethyl citrate by liver homogenates and blood serum from humans, rats, and mice.[20] In the cited article, the authors proposed that pancreatic lipase, rather than liver esterases, was involved as suggested by the pH optimum of 9 for the reaction. *In vivo* studies have indicated reversibility of symptoms and apparent complete recovery from sublethal doses as further evidence of biological degradation.[8,9]

TABLE I
Effect of Pretreatment with Triethyl Citrate on the Growth Inhibitory Action of the Agent

Concentration, moles/l	Per cent growth S.D. from 4–7 replicate cultures: Pretreated cultures with 9 $\times 10^{-3}$ moles/l of triethyl citrate	Untreated cultures (control)
0	100.0 ± 13.3	100.0 ± 11.6
3.29 $\times 10^{-3}$	82.5 ± 8.9	83.6 ± 7.5
6.59 $\times 10^{-3}$	46.2 ± 6.1	45.1 ± 9.3
9.88 $\times 10^{-3}$	7.6 ± 4.9	15.1 ± 2.8
ID_{50}	0.00618 ± 0.00053[a]	0.00639 ± 0.00027

[a] Not significantly different from ID_{50} from untreated samples ($P = 0.05$).

III. SUMMARY AND CONCLUSIONS

The rapid increase in the use of man-made materials in a variety of medical and para-medical applications has necessitated the establishment of testing programs to ensure the safety of the item for an intended use. It also appears that there is a need for very sensitive techniques to appraise in a quantitative manner agents which might be added to man-made materials in order to impart certain desirable properties to the final device or item.

Methodology is described which may be conveniently and inexpensively used to examine many types of chemical compounds for toxicity potential or cellular interferences. Cell populations were determined by simple extraction techniques with subsequent spectrophotometric procedures for the determination of total nucleic

acids. Cultures were shown to be sensitive to small changes in chemical concentration, yielding reproducible and highly quantitative data.

Triethyl citrate demonstrated growth inhibitory properties which appeared quite different from those exhibited by sodium citrate or ethanol. Calcium chloride reversed the action of sodium citrate but had little or no effect on the inhibitory properties of triethyl citrate. Ethanol had no effect in the concentration range equivalent to that when the ester would be completely hydrolyzed. Resistance to triethyl citrate was not seen among cells previously cultured in the presence of the ester. The results of the experiments suggested that the ester is detoxified with time in culture.

The cell culture method described in this paper is now being applied to other compounds used in polymeric materials and will be reported in subsequent papers. Up to this time the usefulness of the method has been very encouraging and should lead to practical applications as a routine method of testing for toxicity as well as to theoretical approaches for suggesting the mechanistic view for the toxicity.

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Received November 17, 1966

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TOXICOLOGICAL STUDIES ON ISOPROPYL AND STEARYL CITRATES[a, b]

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(Manuscript received January 6, 1951)

In view of the increased demand for margarine in recent years, a need has developed for new sources of edible vegetable oils for hydrogenation. Since the production of soybean oil has been markedly increased in the United States within the last decade, the margarine industry has naturally turned to this oil to supplement the supply of other vegetable oils. Thus, while 87 million pounds of soybean oil were used in 1941 for hydrogenation, out of a total of 257 million pounds of all vegetable oils, the quantity of this oil employed in a 12-month period, ending June 30, 1950, was reported as 233 million pounds out of a total of 701 million pounds used by the margarine industry. In other branches of the edible fat industries, there has also been an increased usage of soybean oil.

The chief difficulty in the use of soybean oil is its property of undergoing a so-called "flavor reversion." This change results in the development of an unpleasant taste. The reversion begins in both limpid and hydrogenated oils almost immediately after deodorization. According to Martin, Schepartz, and Daubert *(20)*, Schepartz and Daubert *(30)*, and Stapf and Daubert *(32)* this reaction is associated with the production of carbonyl compounds.

Citric acid is especially satisfactory as an anti-flavor-reversion agent. However, neither citric acid nor its salts dissolve in sufficient amounts in oil to be used conveniently for this purpose.

Vahlteich, Gooding, and Neal in rotating authorship *(8, 9, 10, 11, 22, 23, 24, 35, 36, 37, 38, 39)* have recently reported the synthesis and effectiveness of fat-soluble esters of citric acid in inhibiting the flavor-reversion of soybean oil, and in stabilizing the flavor of other fatty foods. The compounds were mixed mono-, di-, and tri-esters of citric acid, containing from 3 to 18 carbon atoms in the alcohol moiety. The mono-esters were the effective agents in stabilizing oil flavor while the di-esters, particularly the higher homologues, exhibited in addition emulsifying properties.

Before the above-mentioned esters of citric acid can enjoy any widespread application in foods, it is necessary to prove that they are not toxic.

Obviously, it is not possible to test in a thorough manner all of the esters of citric acid mentioned in the patents cited. The lowest and highest homologues in the series were subjected to exhaustive toxicological and metabolic studies. The lowest homologue selected was the mixture of

[a] Supported by a grant from The Best Foods, Inc. Some of these results were reported at the joint meeting of the Northern and Southern California Sections of the Institute of Food Technologists in February, 1949.

[b] Contribution No. 275 of the Department of Biochemistry and Nutrition.

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For this purpose, a mixture of
table oil is most desirable *(39)*
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isopropyl citrate esters, predominantly the mono-ester, and the highest homologue selected was the mixture of stearyl citrate esters, predominantly the di-ester, having a melting point of 57 to 58°C. By the above it is not meant that the toxicity studies represent a blanket investigation of all homologues between the isopropyl and stearyl citrate esters. The basis of selection of the lowest homologue was because of its greatest effectiveness per unit weight in stabilizing the flavor of oils. The stearyl esters were chosen because these were far more readily soluble in oils than the lower members of the series. Indeed this same reasoning constitutes the bases for selecting these two types of esters in commercial application *(25)*.

As far as the hydrolysis products of these selected esters of citric acid are concerned, there is no danger of toxicity, even for a consumption of many times the levels required to preserve the oils. Both hydrolysis products of stearyl citrate esters are components of animal tissues, and are produced by normal metabolic processes. Not only are citrates widely distributed components of foods such as the citrus fruits and milk, but they are also intermediary products of carbohydrate metabolism in the so-called citric acid cycle *(14, 33)*. At the present time, the use of "citric acid and harmless citrates" is permitted in margarine without any specified limit, but only when used for the purpose of producing diacetyl *(6)*.

Stearyl alcohol has been found by Schoenheimer and Hilgetag *(31)* to occur in the feces of dogs; cetyl alcohol, however, was found to be a normal constituent of the feces of dogs, cats, and men. In fact, Stetten and Schoenheimer *(34)* have demonstrated that an interconversion of stearyl alcohol and stearic acid is a usual metabolic process. Moreover, stearyl stearate has been shown by Hilditch *(12)* to account for 2.5% of the total lipid in the head oil of the sperm whale.

Although isopropyl alcohol has toxicological properties at relatively high levels, no effects of this compound have been demonstrated which do not also result from the ingestion of ethyl alcohol *(16)*. Isopropanol has been shown to be approximately twice as toxic as is ethyl alcohol *(18)*. However, doses from 0.75 to 5.28 ml. of isopropanol per kg. body weight per day have been administered to rats without the development of any untoward symptoms. Administration of this alcohol over prolonged periods failed to show evidence of any delayed toxic effects *(16)*. It was likewise found that no deleterious effects on growth, pregnancy, or lactation resulted over several generations in rats given considerable quantities of isopropanol daily *(19)*.

The isopropyl citrates are dispersible in fat with some difficulty whereas the stearyl citrates are readily soluble in fat. For this reason, the first type of citric acid ester is introduced into an oil in a suitable vehicle. For this purpose, a mixture of mono- and diglycerides (1:1) of a vegetable oil is most desirable *(39)*.

The mixture of mono- and diglycerides which is used in conjunction with isopropyl citrate cannot be considered to be a substance possessing toxicity. Mono- and diglycerides occur physiologically in the digestion of fats *(7)*. Alpha-monopalmitin has been identified as a minor constituent of adrenal lipids *(29, 42)*, while Jones, Koch, Heath, and Munson *(13)* have recently reported the isolation of this monoglyceride from normal

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TABLE 1

Typical Compositions of Compounds Investigated

Substance	Melting point, °C.	Acid value	Saponification value	Composition	Typical analyses[c]		
					Isopropyl alcohol[d]	Citric acid[e]	Stearyl alcohol[f]
					%	%	%
Isopropyl citrates in a vehicle consisting of mono- and diglycerides (1:1) of vegetable oil.	Liquid	160	370	27% mono-isopropyl citrate[g]	10.9	26.6	.....
				9% di-isopropyl citrate	(11.0)	(30.0)	.....
				2% tri-isopropyl citrate			
				62% mono- and diglycerides			
Stearyl citrates	57.8	88.6	248.1	12.5% mono-stearyl citrate[h]	.....	25.5	77.9
				75% distearyl citrate	.....	(28.3)	(76.8)
				12.5% tristearyl citrate			

[c] Figures in parentheses are calculated values.
[d] Isopropyl alcohol was determined after oxidation to acetone by refluxing with aqueous potassium dichromate and by precipitation with Deniges reagent *(40)*.
[e] Citric acid was determined on the aqueous extract after saponification by the method of Pucher, Wakeman, and Vickery *(28)* as modified by Perlman, Lardy, and Johnson *(27)*.
[f] Stearyl alcohol was determined as NSF by purification of the residue after saponification by filtration, drying, re-solution, and removal of the solvent.
[g] The 38% of isopropyl citrates is composed of about 65-80% mono-isopropyl citrates, 15-30% of di-isopropyl citrates and 5-10% tri-isopropyl citrate. Typical acid and saponification values are 410 and 690 respectively.
[h] The stearyl citrates are composed of about 10-15% monostearyl citrate, 70-80% distearyl citrates, and 10-15% tristearyl citrates.

The most important factors which determine the behavior of a substance in the body are its digestibility and absorption. If a substance is non-absorbable and at the same time non-irritating to the gastrointestinal tract, there is little possibility that it will exhibit any toxic effects. Data on the absorption and digestibility of isopropyl citrates, stearyl alcohol, and stearyl citrates have been presented in another communication *(3)*.

It was found that IC plus Vehicle in large amounts did not lower the high digestibility of margarine fat when fed to the rat or dog. On the other hand, when SC was fed at very high levels to the rat, the digestibility of the margarine fat was somewhat depressed. However, when the SC was given at a level of 3 times the maximal proposed level *(25)*, assuming that all the fat in the diet was so treated, the digestibility of the margarine fat was not appreciably altered *(3)*. In the case of the dog, no significant depression in digestibility of the margarine fat occurred when the level of SC in the fat was fully 80 times the maximal proposed level.

EXPERIMENTAL

In order to check for any possible toxicological effects of isopropyl citrate esters, predominantly mono-isopropyl citrate (IC), the same plus the mono- and diglyceride vehicle (IC plus Vehicle), and of stearyl citrate esters, predominantly distearyl citrate, (SC), tests have been carried out on rats, rabbits, and dogs. The use of the several species in such studies is in line with the suggestions of Lehman, Lang, Woodard, Draize, Fitzhugh, and Nelson *(17)* that at least 2 species (including one which is a non-rodent) be employed.

All the rats used in the several types of experiments were from our stock colony. LD_{50} studies were conducted on IC, IC plus Vehicle, and on SC.

Acute tests were made on male and female rats at 3 levels for each test substance for a 6-week period. Control experiments in which no test substance was included in the diets were carried out in each series. In most cases, 10 rats of each sex were used per group.

The third type of test (chronic) employed with the rats was essentially a longevity experiment carried out over a period of 2 years with the same distribution in groups as in the acute tests. In both the acute and the chronic tests, the animals were sacrificed at the end of the experiment, and tissues were obtained for histopathological examination.

Another procedure used here for the detection of any possible chronic or cumulative toxicity includes multigeneration studies. These involved raising the first generation of rats from weaning on the several test diets, and on a control diet, and continuing them on these regimens during the breeding and the lactation periods. When the young were weaned, the parents were discarded and the second generation was continued on the same diets which the parents had received. A similar routine was employed for succeeding generations through the weaning of the sixth generation, in the IC plus Vehicle tests, and of the fifth generation in the tests with SC.

In still another approach, additional groups of rats were used to ascertain whether these esters of citric acid in oils develop a toxicity when heated at 205°C. (a temperature used in deep frying) over a prolonged period (8 hrs.). The oils previously heated with or without the citric acid ester, as well as potato chips prepared by frying in such oils, were incorporated in diets which were then tested by growth studies on weanling rats over 11-week periods.

In the complete series of tests reported here (not including the absorption and digestibility studies), a total of 2398 rats was used.

Rabbits and dogs were employed for acute toxicity studies on the citric acid esters. The rabbit tests were continued for 6 weeks, while the experiments on dogs lasted for a 12-week period. A histopathological examination was made of the tissues of all of the test animals at the conclusion of the experimental period. In addition, massive single doses of IC plus Vehicle or of SC were administered to dogs. A total of 57 rabbits and 24 dogs was used in the toxicity tests.

The LD_{50} tests were carried out by the administration of the test substance by stomach tube to the fasted animals. In the rat tests, studies were made on IC, an equivalent isopropanol-citric acid mixture as control, IC plus Vehicle alone and in cottonseed oil, cottonseed oil alone as a control, and SC in cottonseed oil. The tests were considered positive if the animals died within periods of 4 to 6 days; this interval was chosen since, at that time, practically all the animals remaining alive were again gaining weight and in good nutritional condition. The dog experiments were carried out in essentially the same manner but because of necessity the number of test animals was restricted.

The diets used in the rat and dog experiments were made up of purified foodstuffs. The compositions of the various diets used are listed in Tables 2 and 3.

The rats used on the LD_{50} tests had been maintained on our usual stock diet. They were fasted for 18 hours prior to the administration of the test substance by stomach tube.

Rabbits were fed throughout the tests on commercial rabbit pellets.[9] IC plus Vehicle was mixed with the rabbit pellets in amounts of 0, 1.9, 3.8, 7.5, or 15.0% (0.0, 0.72, 1.4, 2.8, or 5.7% IC, respectively). In the tests with SC, the pellets were mixed with 2 or with 11% of the melted substance. The diets fed the dogs in the 3-month acute tests are patterned after those described by Cowgill *(4)*. Table 3 gives the composition of these diets.

The IC, IC plus Vehicle, and SC employed in the tests reported here were commercial products supplied by The Best Foods, Inc. The identity of the products was confirmed by our analysis. Hemoglobin determinations were made on the dogs by the acid hematin method.

[9]Albers Rabbit Family Ration.

TABLE 2

The Composition of Diets Used in the Rat Tests

Dietary component	Acute tests					Chronic tests			Multigeneration tests				Heated oil tests		
	70	80	81	82	83	84	85	86	71	72	87	88	73	89	74	90
Isopropyl citrate esters, predominantly mono-isopropyl plus mono- and diglyceride vehicle (IC plus Vehicle)																
	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
Casein	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0
Sucrose	55.5	54.9	54.3	53.0	50.5	55.5	55.5	55.5	50.5	53.5	50.5	53.5	55.5	55.5	44.0	44.0
Whole margarine[l]	12.50	11.42	10.34	8.00	3.50	12.22	11.94	9.70	12.5	12.5	9.7	9.7	....	....	....	....
Margarine fat	....	....	....	....	....	....	....	....	....	....	....	....	11.5[j]	11.44[j]	....	....
Fat-soluble vitamin mixture[k]	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....
Yeast[l]	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	1.0	1.0	1.0	1.0
Salt mixture[m]	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	8.0	8.0	8.0	8.0
Liver powder[n]	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....

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	70	80	81	82	83	84	85	86	71	72	87	88	73	89	74	90
Isopropyl citrate esters, predominantly mono-isopropyl plus mono- and diglyceride vehicle (IC plus Vehicle)																
	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
Casein	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0
Sucrose	55.5	54.9	54.3	53.0	50.5	55.5	55.5	55.5	50.5	53.5	50.5	53.5	55.5	55.5	44.0	44.0
Whole margarine[i]	12.50	11.42	10.34	8.00	3.50	12.22	11.94	9.70	12.5	12.5	9.7	9.7	....	....	....	....
Margarine fat	....	....	....	....	....	....	....	....	....	....	....	....	11.5[j]	11.44[j]	....	....
Fat-soluble vitamin mixture[k]	....	....	....	....	....	....	....	....	....	....	....	....	1.0	1.0	1.0	1.0
Yeast[l]	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0
Salt mixture[m]	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Liver powder[n]	....	....	....	....	....	....	....	....	5.0[o]	2.0[p]	5.0[o]	2.0[p]	....	....	....	....
Potato chips:																
Fried in margarine fat	....	....	....	....	....	....	....	....	....	....	....	....	....	....	23.0	....
Fried in margarine fat containing IC plus Vehicle (0.5%)	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	23.0
IC plus Vehicle	....	1.68	3.36	7.00	14.00	0.28	0.58	2.80	....	....	2.8	2.8	....	0.06	....	....
IC content	....	0.64	1.3	2.7	5.3	0.11	0.21	1.1	....	....	1.1	1.1	....	0.02	....	....
Stearyl citrate, predominantly distearyl citrate (SC)																
Dietary component	170	180	181	182	183	184	185	186	171		187	188	73	189	74	190
	%	%	%	%	%	%	%	%	%		%	%	%	%	%	%
Casein	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	19.0		19.0	19.0	20.0	20.0	20.0	20.0
Sucrose	55.5	54.2	53.0	50.5	45.5	55.0	53.5	45.5	51.8		51.0	43.3	55.5	55.5	44.0	44.0
Margarine fat	11.5	11.5	11.5	11.5	11.5	11.5	11.5	11.5	11.0		9.9	10.0	11.5[j]	10.64	....	....
Fat-soluble vitamin mixture[k]	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0		1.0	1.0	1.0	1.0	1.0	1.0
Yeast[l]	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	7.6		7.6	7.6	8.0	8.0	8.0	8.0
Salt mixture[m]	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.8		4.8	4.8	4.0	4.0	4.0	4.0
Liver powder[n,o]	....	....	....	....	....	....	....	....	4.8		4.8	4.8	....	....	....	....
Potato chips:																
Fried in margarine fat	....	....	....	....	....	....	....	....	....	....	....	....	....	....	23.0	....
Fried in margarine fat containing SC (7.5%)	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	23.0
SC	....	1.3	2.5	5.0	10.00	0.5	2.0	10.0	....		1.9	9.5	....	0.86	....	....

[i] 8 I U of vitamin D (as viosterol) added per g. food. [j] Heated for 8 hours at 205°C. [k] Composition was as follows: α-tocopherol, 0.5 g.; carotene (General Biochemicals, Inc.), 20 mg.; vitamin D_2 (crystalline Winthrop), 0.5 mg.; cottonseed oil to make a total weight of 100 g. [l] Anheuser-Busch, Strain G. [m] Osborne-Mendel salt mixture (*26*). [n] Wilson Laboratories. [o] 1:20 concentrate. [p] 1:50 concentrate.

TABLE 3

Composition of the Diets Used in the Experiments on Dogs

Food component	Composition of diet		
	D-1	D-2	D-3
	%	%	%
Commercial casein	20.0	20.0	20.0
Sucrose	35.0	35.0	35.0
Dextrin	12.2	12.2	12.2
Margarine oil[r]	18.3	18.24	15.3
Salt mixture (Reference 41)	2.0	2.0	2.0
Cellu flour[s]	2.5	2.5	2.5
Cod-liver oil (U.S.P.)	5.0	5.0	5.0
Dried brewers' yeast[t]	5.0	5.0	5.0
IC plus Vehicle	....	0.06	....
SC	....	....	3.0

[r] Without additives. [s] Chicago Dietetics Supply House. [t] Anheuser-Busch (Strain G).

RESULTS AND DISCUSSION

LD_{50} Studies

The acute toxicity, as revealed by the determination of the LD_{50} levels, has been ascertained for rats, and checked with dogs. The results of the tests on rats are summarized in Table 4.

The LD_{50} values of the several test materials as determined on rats are all quite high. The LD_{50} value with male rats for IC plus Vehicle, when given alone, exceeded 20.7 g./kg., while when it was administered in a ratio of one part of cottonseed oil to 2 parts of IC plus Vehicle, the value was greater than 17.2 g./kg. The corresponding figures for the female rats were 18.8 and 19.2 g./kg. This would indicate that supplementation of IC plus Vehicle with fat does not accentuate the LD_{50} figure.

When IC was administered in alcohol solution or in water, the toxicity was 2.8 to 3.7 g./kg. The slightly lower LD_{50} value for the females receiving IC in ethanol may be the reflection of the higher alcohol dose which was administered. In the tests with females where an aqueous solution was employed, the figure for LD_{50} approximated the value obtained for the males. The quantity of IC required to produce 50% mortality approximated that for its hydrolysis products. However, when IC is administered in a fat vehicle, more than double the amount of IC is required to produce a 50% mortality.

The LD_{50} level for SC exceeds 5.4 g./kg. in the case of both male and female rats. Since it was impossible to prepare a solution of SC, liquid at body temperature, in a higher concentration than 20%, the maximum dose (30 ml./kg.) which could be introduced into the stomachs of rats contained only 5.4 g./kg.

Twelve dogs were used to check the LD_{50} values obtained with rats. Massive single doses, viz. 12 g./kg. of IC plus Vehicle, 2.25 g./kg. of IC, and 5 g./kg. of SC, were not fatal to the dog. After a period of 2 days, the dogs were eating normally and showed no obvious ill effects from the dosage.

Substance tested	S r
IC plus Vehicle	
Dosage (g./kg.)	
Number of rats	
Mortality (%)	
Dosage (g./kg.)	
Number of rats	
Mortality (%)	
IC plus Vehicle in cottonseed oil (2:1)	
Dosage (g./kg.)	M
Number of rats	
Mortality (%)	
Dosage (g./kg.)	F
Number of rats	
Mortality (%)	
IC	
75% in 10% ethanol	
Dosage (g./kg.)	M
Number of rats	
Mortality (%)	
15% in 10% ethanol	
Dosage (g./kg.)	F
Number of rats	
Mortality (%)	
75% in water	
Dosage (g./kg.)	F
Number of rats	
Mortality (%)	
Isopropyl alcohol plus citric-acid in ethanol	
Dosage (g./kg.)	F
Number of rats	
Mortality (%)	
SC (20% in cottonseed oil)	
Dosage (g./kg.)	M
Sol. vol. (ml./kg.)	
Number of rats	
Mortality (%)	
Number of rats	F
Mortality (%)	

[a] Figures in parentheses represe

S

Experiments on Rats. T
incorporated in the comme
data. No animals maintaine
the tests.

There is no evidence, on
either IC plus Vehicle or S

TABLE 4

LD_{50} Tests on Rats

Substance tested	Sex of rats	Data on different levels of tests						Estimated LD_{50}[a]
IC plus Vehicle								*g./kg.*
Dosage (g./kg.)	M	2.1-20.7	.....	.....	.....	.....	.....	>20.7
Number of rats		6	.....	.....	.....	.....	.....	(>7.9)
Mortality (%)		0	.....	.....	.....	.....	.....	.....
Dosage (g./kg.)	F	2.7-15.5	17.7	21.0	23.6	27.0	.....	18.8
Number of rats		4	10	10	11	10	.....	(7.2)
Mortality (%)		0	30	90	89	100	.....	.....
IC plus Vehicle in cottonseed oil (2:1)								
Dosage (g./kg.)	M	0	11.1	14.1	16.1	17.2	.....	>17.2
Number of rats		10	9	10	9	9	.....	(>6.5)
Mortality (%)		0	11	20	22	33	.....	.....
Dosage (g./kg.)	F	0	12.0-13.4	15.8	17.9	19.2	22.2	19.2
Number of rats		10	20	10	10	10	10	(7.3)
Mortality (%)		0	0	20	10	50	50	.....
IC								
75% in 10% ethanol								
Dosage (g./kg.)	M	2.1	2.7	3.3	3.9	.....	.....	3.7
Number of rats		10	10	10	10	.....	.....	.....
Mortality (%)		0	20	10	70	.....	.....	.....
15% in 10% ethanol								
Dosage (g./kg.)	F	2.1	2.7	3.3	3.9	.....	.....	2.8
Number of rats		10	10	10	10	.....	.....	.....
Mortality (%)		10	40	90	100	.....	.....	.....
75% in water								
Dosage (g./kg.)	F	2.25	3.0	3.75	4.5	5.25	5.25[w]	3.6
Number of rats		10	10	10	10	10	10	.....
Mortality (%)		0	10	60	70	100	90	.....
Isopropyl alcohol plus citric-acid in ethanol								
Dosage (g./kg.)	F	3.9[v]	3.9	5.25[v]	5.25[w]	.....	.....	4.5
Number of rats		10	9	10	10	.....	.....	.....
Mortality (%)		0	22	90	100	.....	.....	.....
SC (20% in cottonseed oil)								
Dosage (g./kg.)	M	0	0.9-1.8	2.7	3.6	4.5	5.4	>5.4
Sol. vol. (ml./kg.)		30	5-10	15	20	25	30	.....
Number of rats		10	4	2	12	13	10	.....
Mortality (%)		0	0	0	8	15	10	.....
Number of rats	F	10	4	2	12	13	10	>5.4
Mortality (%)		0	0	0	0	0	0	.....

[a] Figures in parentheses represent IC content of mixtures. [v] Male rats. [w] In water.

Short-Term (Acute) Tests

Experiments on Rats. Tests were made on IC plus Vehicle or SC when incorporated in the commercial margarine fat. Table 5 summarizes these data. No animals maintained on any of the diets died during the course of the tests.

There is no evidence, on the basis of the acute tests on adult rats, that either IC plus Vehicle or SC possesses any degree of toxicity. Thus, adult

TABLE 5

The Change in the Weight of Adult Rats Receiving Control Diets Alone or Similar Diets to Which Several Levels of IC plus Vehicle or SC Were Added

Diet no.	Test material in diet	Experiments on male rats						Experiments on female rats					
		Number	Body weight		Increase in weight	Total food eaten	Total test substance eaten	Number	Body weight		Increase in weight	Total food eaten	Total test substance eaten
			Start	End					Start	End			
Isopropyl citrate esters, predominantly mono-isopropyl citrate, plus mono- and diglyceride vehicle (IC plus Vehicle)													
	%		g.	g.	g.	g.	g.		g.	g.	g.	g.	g.
70	0.0	10	336	386	50.2±2.7	625	0	10	221	247	26.5±3.5	496	0
80	1.68(0.64)*	10	326	364	38.3±5.3	568	9.54	10	208	230	21.8±4.6	447	7.51
81	3.36(1.8)	10	324	368	44.8±4.9	588	19.8	9	214	231	17.3±5.1	450	15.1
82	7.00(2.7)	9	331	373	42.0±6.7	597	41.8	10	210	226	15.5±3.5	437	30.6
83	14.00(5.3)	10	329	372	42.6±4.1	615	86.1	10	208	223	15.0±7.8	432	60.5
Stearyl citrate esters, predominantly distearyl citrate (SC)													
170	0	10	203	271	67.9±5.9	442	0	10	202	230	27.6±3.4	390	0
180	1.3	10	206	302	96.0±7.9	514	6.68	10	202	230	28.4±3.6	422	5.48
181	2.5	10	209	292	82.7±7.5	494	12.3	10	206	235	29.2±3.8	406	10.1
182	5.0	10	205	296	90.8±7.8	542	27.1	10	196	220	25.0±5.5	447	22.3
183	10.0	10	208	304	95.4±6.9	555	55.5	10	193	220	27.2±5.2	445	44.5

* IC content of IC plus Vehicle in parentheses.

male rats receiving IC plu
this ester over a 6-week P
female rats ingested an av
per rat per day) without
respond to a daily intake
rat per day in the 2 sexes.

The results of the acut
plus Vehicle. The average
the highest dosage was 55.5
by the females was 44.5 g.

The slight average diffe
between the control animal
doses of IC plus Vehicle a
on adult rats of about 6 mo
parallelism with dosage. Li
ently better growth respon
compared to the responses

Experiments on Rabbits.
on rabbits receiving the IC
animals were fed on the ba
was administered daily to
period. In the second and th
and in all the tests SC
with the crushed rabbit pell
Table 6 shows the average r

The Change in Weight of Rabbit
Levels of IC plus Vehicle
or SC Mixe

Group No.	Series	Test material in diet	Number	Sh
IC				
		%		g
1	I	0	2	20
2	I	2.2	3	20
3	I	4.4	2	23
4	I	9.2	1	21
IC plus				
5	III[7]	0	8	25
6	II	1.9	1	20
7	II	3.8	2	20
8	II	7.5	2	25
9	II	15.0	5	21
10	III[7]	11.3	8	24
11	III	22.5	3	24
S				
12	III	2	8	25
13	III	10	3	25

[7] One rabbit died during

male rats receiving IC plus Vehicle at the highest level ingested 86 g. of this ester over a 6-week period (more than 2 g. per rat per day), and female rats ingested an average of 60 g. over the same period (1.42 g. per rat per day) without any demonstrable harmful effects. These correspond to a daily intake of 0.78 g. and 0.54 g. respectively of IC per rat per day in the 2 sexes.

The results of the acute tests on SC are quite similar to those on IC plus Vehicle. The average level of SC taken in by the male rats receiving the highest dosage was 55.5 g. (1.32 g. per rat per day) and that ingested by the females was 44.5 g. (1.06 g. per rat per day).

The slight average differences noted in the increases in body weight between the control animals and those receiving progressively increasing doses of IC plus Vehicle are not significant. These tests were conducted on adult rats of about 6 months of age and the growth responses show no parallelism with dosage. Likewise, no significance is attached to the apparently better growth responses of the adult animals receiving SC when compared to the responses of the control rats.

Experiments on Rabbits. Two types of experiments were carried out on rabbits receiving the IC plus Vehicle. In the first series of tests, the animals were fed on the basal diet (rabbit pellets) and the test substance was administered daily to the animals by stomach tube over the 6-week period. In the second and third series of experiments with IC plus Vehicle, and in all the tests with SC, the substances under examination were mixed with the crushed rabbit pellets before this material was fed to the rabbits. Table 6 shows the average results of these tests.

TABLE 6

The Change in Weight of Rabbits Receiving Control Diets or Those Containing Several Levels of IC plus Vehicle by Stomach Tube (Series I), or IC plus Vehicle or SC Mixed with Diet (Series II and III)

Group No.	Series	Test material in diet	Number	Body weight			Average gain in weight	Average total food eaten	Average test material given
				Start	3 weeks	6 weeks			
IC plus Vehicle by stomach tube									
		%		g.	g.	g.	g.	g.	g.
1	I	0	2	2010	2204	2770	760	Ad libitum	0
2	I	2.2	3	2098	2407	2899	801	food intake	120
3	I	4.4	2	2300	2598	2885	585	not record-	240
4	I	9.2	1	2104	2380	2780	676	ed.	500
IC plus Vehicle incorporated in diet									
5	III[y]	0	8	2503	2821	3263	760	5413	0
6	II	1.9	1	2092	2402	2735	641	5411	113
7	II	3.8	2	2026	2456	2985	959	5399	205
8	II	7.5	2	2550	2632	2902	352	5010	376
9	II	15.0	5	2166	2556	2927	761	5366	805
10	III[y]	11.3	8	2427	2952	3276	849	5175	585
11	III	22.5	8	2442	2702	3039	597	3803	856
SC incorporated in diet									
12	III	2	8	2532	2729	3171	640	4763	95.3
13	III	10	8	2516	2599	2950	434	4428	442.8

[y] One rabbit died during course of test.

Only 2 deaths occurred among the 57 rabbits during the course of the test. One of these was in Group 5 (control) and the second was in Group 10 (receiving 11.3% of the composite, IC plus Vehicle). The average intake of IC plus Vehicle of 4 groups of rabbits (22 animals) exceeded 500 g. during the 6-week period. The maximum consumption for the period was by one rabbit in Group 11. The total amounted to 1045 g., or 24.9 g. daily; this approximates a daily intake of IC plus Vehicle of 1% of the body weight.

The intake of the SC was somewhat lower, averaging 443 g. for the group on the higher dosage. The highest consumption in this group was 513 g. of the test substance for the period, or 12.2 g. per rabbit per day.

The average gain in weight for the control rabbits was 760 g. for the 6-week period. Several groups receiving IC plus Vehicle (Groups 2, 7, 9, 10) equalled or exceeded this figure. In both groups receiving SC, the average gain in weight was somewhat less. All rabbits receiving SC showed excellent increase in weight, except one animal which lost weight during the early part of the test when it failed to eat; this rabbit began to regain its weight during the last week of the test. It had returned to approximately its starting weight at the termination of the experiment.

A histopathological examination was made on 311 sections of 284 organs from 24 rabbits by one of us (H.R.F.). These included sections of the liver, kidney, heart, brain, lung, spleen, stomach, small intestine, large intestine, pancreas, adrenal, and testicle of the 8 rabbits receiving the highest dose of IC plus Vehicle (22.5%), of the 8 animals given higher dose of SC (10%), and of the 8 controls (Group 5) in Series III. These were all males. There was no obvious morphological evidence of disease in the tissues of any group given diets in the categories listed.

Experiments on Dogs. Acute tests were made on 12 dogs over 11 to 12-week periods. Three groups of 4 each were used. The dietary regimens included a basal diet (control group), a similar diet including 0.06% IC plus Vehicle, and a third to which 3.0% of SC was added. The dogs used consisted of a litter of 6 cocker puppies, which were distributed equally between the groups, and 6 adult mongrels of unknown nutritional history. The adults were devocalized and dewormed before the test was started, while the puppies were devocalized during the second week of the tests. Because of the uniformity in origin and dietary background of the puppies, greater significance should be attached to weight changes of these animals.

All dogs received measured amounts of food once daily. In the case of the adult dogs, the amounts were first based on the quantities necessary to establish an optimum body weight, as calculated from Cowgill's formula *(5)*. When this weight was attained, they were placed on a maintenance diet. In the case of the puppies, the diet was adjusted to give a steady rate of growth. Water was given ad libitum throughout. The data on these tests are included in Table 7.

There is no evidence of toxicity of IC plus Vehicle or SC when fed to dogs over a 12-week period. IC plus Vehicle was fed at a level of 0.25% of the fat, which is 10 times the level proposed for its use in margarine (0.02% of the whole margarine or 0.025% of the margarine fat). SC was fed at a level of 14.8% of the fat, which is 80 times the maximum level to

TABLE 7

The Change in Weight of Dogs Receiving a Control Diet or Diets Containing IC plus Vehicle or SC

Dog No.	Type of dog	Sex	Body weight				Gain in weight	Total food eaten	Total test substance eaten	Hemoglobin at end of test
			Start	After 4 weeks	After 8 weeks	After 12 weeks				
Control group (Diet D-1)										
			kg.	kg.	kg.	kg.	kg.	g.	g.	
1	Cocker pup	M	2.3	3.2	3.[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

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ing 443 g. for the
in this group was
per rabbit per day.
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receiving SC showed
lost weight during
bbit began to regain
returned to approxi-
periment.

ections of 284 organs
sections of the liver,
stine, large intestine,
g the highest dose of
r dose of SC (10%),
were all males. There
n the tissues of any

12 dogs over 11 to
The dietary regimens
including 0.06% IC
dded. The dogs used
e distributed equally
n nutritional history.
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r its use in margarine
margarine fat). SC was
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TABLE 7

The Change in Weight of Dogs Receiving a Control Diet or Diets Containing IC plus Vehicle or SC

Dog No.	Type of dog	Sex	Body weight: Start	Body weight: After 4 weeks	Body weight: After 8 weeks	Body weight: After 12 weeks	Gain in weight	Total food eaten	Total test substance eaten	Hemoglobin at end of test
			Control group (Diet D-1)							
			kg.	*kg.*	*kg.*	*kg.*	*kg.*	*g.*	*g.*	%
1	Cocker pup	M	2.3	3.2	3.5	3.6	1.3	7990	.....	12.9
2	Cocker pup	F	2.0	2.8	3.2	3.2	1.2	7927	.....	14.2
3	Mongrel adult	M	15.9	18.2	18.5	18.8[a]	2.9	27230	.....	15.9
4	Mongrel adult	M	13.5	15.4	15.9	16.3	2.8	16870	.....	17.6
Average[aa]			2.15	3.00	3.35	3.40	1.25	7958	.....	.....
			Group receiving IC plus Vehicle (Diet D-2)							
5	Cocker pup	F	2.3	3.3	3.7	3.8	1.5	7918	4.75	12.6
6	Cocker pup	F	2.5	3.1	3.6	3.8	1.3	7663	4.60	14.0
7	Mongrel adult	M	10.9	12.8	14.8	15.0[a]	4.1	23330	13.98	14.3
8	Mongrel adult	F	5.0	6.4	6.8	7.4	2.4	17290	10.37	18.6
Average[aa]			2.40	3.20	3.65	3.80	1.40	7790	4.68	.....
			Group receiving SC (Diet D-3)							
9	Cocker pup	F	2.1	3.2	3.7	3.8	1.7	7683	230.5	12.9
10	Cocker pup	F	1.8	2.6	3.2	3.3	1.5	7887	236.6	12.8
11	Cocker adult	M	12.7	15.5	17.1	17.3[a]	4.6	19320	579.6	13.6
12	Mongrel adult	M	5.2	4.8	5.0	5.7	0.5	7990	239.7	14.0
Average[aa]			1.95	2.90	3.45	3.55	1.60	7785	233.6	.....

[a] Weight at 11 weeks. [aa] Average of litter mate cocker puppies only.

be used in margarine (0.15% of the whole margarine or 0.19% of margarine fat).

The amount of food consumed in the 3 groups was comparable. Actually, in the case of the puppies, the gain in weight of the experimental groups somewhat exceeded that of the control group, although the former animals consumed somewhat less food. The same pattern was followed by the adult dogs. Hemoglobin levels determined before the dogs were sacrificed were practically identical for the several groups. A histopathological examination of the livers and kidneys of the dogs failed to reveal any evidence of pathological manifestations attributable to the test substance. Whatever divergencies from normal occurred were distributed equally among the several groups.

Long-Term (Chronic) Tests on Rats

Longevity Tests. In order to determine whether the materials tested may exhibit a toxicological action when their administration is continued over a prolonged period, chronic tests were carried out on rats for 2 years. The tests were discontinued at the end of this interval, so that the tissues could be obtained for pathological examination from animals which had remained alive until the termination of the experiment. Data on the body weights of the rats in the various series of tests at representative periods during the experiment, as well as on their survival, are included in Table 8.

With the exception of the control group in the distearyl citrate tests, 55% or more of the rats were still alive after receiving the various diets for a period of 2 years. When allowances are made for accidental deaths, the percentage survival in the tests with IC plus Vehicle were 59, 82, and 58% for the groups receiving the low, intermediate, or high level of test substance, with the value of 63% for the control group. In the experiments in which SC was added to the diets, the survivals amounted to 66, 65, and 55% for the groups receiving the progressively increasing proportions of the test compound, with an average of 44% for the corresponding control group.

No marked variations in weight between the experimental and the control groups were noted during the 2-year period. However, after 104 weeks, the average of the means of the weights of males and females still surviving in the IC plus Vehicle tests were as follows: Diet 70 (control), 414 g.; Diet 84 (0.28%), 400 g.; Diet 85 (0.56%), 425 g.; and Diet 86 (2.8%), 404 g. In the tests with SC, the corresponding average weights were: Diet 170 (control), 380 g.; Diet 184 (0.5%), 486 g.; Diet 185 (2.0%), 413 g.; and Diet 186 (10%), 388 g.

Histopathological sections of liver, kidney, heart, brain, lung, spleen, stomach, small intestine, large intestine, pancreas, adrenal, and testicle or ovary were examined. In the case of the rats receiving IC plus Vehicle at a 2.8% level, it is reported as follows: "It is my opinion that no specific pathological lesions developed which could be attributed to the product fed."[bb] An examination of the same tissues of a series of rats which had

[bb] Report of Professor Ernest M. Hall, Department of Pathology, School of Medicine, University of Southern California.

TABLE 8

The Body Weights and Survivals of Rats Receiving Control Diets or Diets Containing Several Levels of IC plus Vehicle or SC

Category	IC plus Vehicle tests				SC tests			
	Diet 70	Diet 84	Diet 85	Diet 86	Diet 170	Diet 184	Diet 185	Diet 186
Test substance in diet, %	0	0.28	0.56	2.80	0	0.5	2.0	10.0

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TABLE 8

The Body Weights and Survivals of Rats Receiving Control Diets or Diets Containing Several Levels of IC plus Vehicle or SC

Category	IC plus Vehicle tests				SC tests			
	Diet 70	Diet 84	Diet 85	Diet 86	Diet 170	Diet 184	Diet 185	Diet 186
Test substance in diet, %	0	0.28	0.56	2.80	0	0.5	2.0	10.0
IC content	0	0.11	0.21	1.06	....	....	....	....
Male rats								
Number in group	10	10	10	10	10	9	10	10
Body weight:								
Start, g.	35	36	36	36	35	33	34	32
After 32 weeks, g.	339	362	346	324	361	361	338	328
After 64 weeks, g.	421	471	452	419	423	475	423	423
After 96 weeks, g.	488	546	507	467	471	597	488	474
After 104 weeks, g.	520	495	543	461	454	610	524	482
Rats alive at end	7	5	5	4	3	4	6	5
Female rats								
Number in group	10	10	10	10	10	10	10	10
Body weight:								
Start, g.	40	38	39	39	30	32	32	28
After 32 weeks, g.	228	230	216	222	220	222	208	217
After 64 weeks, g.	287	268	263	255	267	282	272	259
After 96 weeks, g.	308	296	316	328	297	359	294	309
After 104 weeks, g.	309	304	307	348	305	363	302	294
Rats alive at end	5	5	9	7	5	8	7	6
Survival of all rats								
Uncorrected, %	60	50	70	55	40	60	65	55
Corrected for those killed accidentally, %	63	59	82	58	44	66	65	55

received distearyl citrate at a 10% level for 2 years was made by one of us (H.R.F.). Although most of the rats exhibited some diseased tissues characteristic of old age, no specific changes traceable to the diet could be noted. Metastatic calcification and tumor formation were noted in certain tissues examined; however, they are not considered to have special significance, inasmuch as they were noted in the tissues of both test and control rats.

Multigeneration Tests. The most sensitive method for the detection of dietary deficiencies or toxicity involves subjecting several generations of the animals to the test diets. All the factors of stress are brought to bear on the animal in such tests, namely growth, pregnancy, and lactation. Diets which are entirely satisfactory for maintenance, or even for growth, over a single generation may fail to be sufficient for reproduction; moreover, a dietary regimen which serves adequately for both growth and reproduction may be unsatisfactory for lactation. The latter stress offers the most severe test of the nutritional value of a diet.

In the multigeneration tests, weanling male and female rats (Generation 1) whose parents had been on the laboratory diet were raised on the test diets and were bred after 10 weeks (at 13 weeks of age). These rats were continued on the test diets during the subsequent pregnancy and lactation. Young from the first generation (Generation 2) were continued after weaning on the same test diets which their parents had received. At maturity the second-generation rats were bred and a routine similar to the above was repeated. In the case of the rats receiving IC plus Vehicle, the experiment was continued through the weaning of the sixth generation; in the tests with SC, the experiment was terminated when the fifth-generation rats were weaned. Table 9 summarizes the data on the growth of the successive generations, while Table 10 records the information to be gained from the pregnancy and lactation performances.

The multigeneration tests indicate that IC plus Vehicle is completely harmless when incorporated in the diet at a level of 2.8% (equivalent to 1.1% of IC). The rats remained fertile throughout the 5 generations. The total litters cast in the test group comprised 91% of the females bred (59 of 65); this figure was identical with that of the control group (90%, 65 of 72). The body weights of the progeny were likewise similar in the control and experimental groups. At the age of 3 days, the average weights were 7.6 and 7.5 g., while at the age of 21 days the mean values were found to be 34.5 and 35.1 g., respectively.

The results of the growth tests carried on from weaning for the successive 10-week period give added confirmation of the non-toxicity of IC plus Vehicle at the 2.8% level. The average 10-week weight of the fifth generation male and female rats on the test diet (254.7 g., 178.3 g.) not only exceeded the average of the first-generation rats on this test diet for the 10-week period (238.7 g., 176.6 g.) but it was also higher than that of the fifth generation of the control rats (223.4 g., 151.2 g.).

A similar analysis of the data indicates that SC exhibits no toxicity over 4 generations when fed at a level of 1.9% or 9.5% of the diet. The fertility remained high in all groups. The percentages of successful pregnancies were as follows: Diet 171 (control), 93% (55 of 59); Diet 187

TABLE 9

Body Weights of Successive Generations of Rats Receiving Control Diets or Those Containing IC plus Vehicle or SC

Diet No.	Test substance in diet	Generation	Male rats				Female rats			
			Number	Body weights			Number	Body weights		
				Start	After 5 weeks	After 10 weeks		Start	After 5 weeks	After 10 weeks
IC plus Vehicle tests										
				g.	g.	g.		g.	g.	g.

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TABLE 9

Body Weights of Successive Generations of Rats Receiving Control Diets or Those Containing IC plus Vehicle or SC

Diet No.	Test substance in diet	Generation	Male rats				Female rats			
			Number	Body weights			Number	Body weights		
				Start	After 5 weeks	After 10 weeks		Start	After 5 weeks	After 10 weeks
				IC plus Vehicle tests						
	%			g.	g.	g.		g.	g.	g.
71	0	1	10	30.1	158.6	246.9	15	30.5	123.6	175.3
		2	9	38.6	168.5	281.5	15	37.8	137.9	187.3
		3	10	36.5	151.1	243.0	16	34.5	113.2	157.1
		4	14	38.8	153.2	224.3	22	37.4	123.0	159.4
		5	13	37.5	143.8	223.4	26	35.9	113.8	151.2
87	2.8	1	11	30.4	155.2	238.7	15	30.3	133.0	176.6
		2	11	38.5	157.7	255.0	12	34.6	132.8	182.1
		3	17	35.0	134.7	227.5	16	34.5	121.2	174.7
		4	12	39.5	164.4	240.0	18	37.9	124.5	160.4
		5	17	38.7	178.1	254.7	21	37.6	136.3	178.3
				SC tests						
171	0	1	5	37.8	164.0	265.0	10	37.3	135.7	182.5
		2	14	34.0	156.6	243.6	21	35.7	125.2	166.3
		3	16	37.4	168.2	253.5	19	35.8	122.7	163.1
		4	18	36.9	161.7	289.3	22	36.5	126.1	179.0
187	1.9	1	5	38.2	167.4	273.0	10	37.3	132.8	177.5
		2	19	34.9	150.1	245.3	16	33.9	122.0	171.1
		3	14	37.1	175.2	257.8	20	35.5	131.7	172.9
		4	10	40.3	172.9	268.4	16	38.6	129.1	177.8
188	9.5	1	4	36.5	160.5	267.3	10	37.6	131.5	177.4
		2	19	36.6	153.7	246.0	26	32.5	125.3	165.4
		3	10	33.2	162.2	237.1	19	32.7	124.7	166.2
		4	13	43.8	174.9	273.3	19	39.5	132.0	178.8

TABLE 10

Pregnancy and Lactation Response of Successive Generations of Rats Receiving Control Diets or Diets Containing IC plus Vehicle or SC

Diet No.	Test substance added	Parents				Litter					
		Generation	Number of rats bred	Number of litters cast	Average time[cc]	Generation	Average number	Body weight		Total rats weaned	Died between 3rd and 21st day
								3 days	21 days[dd]		
IC plus Vehicle tests											
	%				*days*			*g.*	*g.*		
71	0	1	15	13	31.0	2	8.2	7.6	38.3(49)	70	0
		2	10	10	43.2	3	6.2	7.3	28.1(14)	33	4
		3	10	10	35.4	4	6.9	8.0	38.6(28)	56	3
		4	22	17	34.9	5	6.4	7.7	37.0(21)	55	1
		5	25	25	36.1	6	5.8	7.3	30.4(28)	103+[ee]	1
87	2.8	1	12	12	28.8	2	9.9	7.5	35.8(77)	77	0
		2	8	8	31.4	3	8.3	6.9	34.5(35)	35	9
		3	7	7	38.2	4	6.7	7.7	35.2(14)	46	0
		4	17	17	29.6	5	7.9	8.2	34.3(91)	98	0
		5	21	15	42.2	6	6.3	7.2	35.9(28)	56+[ee]	0
SC tests											
171	0	1	10	9	28.0	2	9.4	6.9	34.0(35)	35	0
		2	14	13	27.3	3	8.3	6.6	34.3(35)	60	3
		3	19	17	27.7	4	7.7	7.2	33.6(35)	65	5
		4	16	16	26.6	5	7.1	6.9	34.5(63)	72	7
187	1.9	1	10	10	26.8	2	6.3	6.7	34.6(21)	41	0
		2	15	15	26.2	3	6.8	7.4	33.5(42)	58	0
		3	15	14	24.4	4	8.1	7.0	36.9(56)	83	3
		4	14	14	31.0	5	8.4	6.8	36.7(56)	71	2
188	9.5	1	11	11	30.2	2	7.6	7.0	34.5(43)	61	2
		2	23	23	26.2	3	8.5	7.2	35.5(105)	117	0
		3	16	14	26.5	4	8.5	8.5	41.0(56)	67	0
		4	14	14	27.4	5	9.4	7.6	37.1(84)	94	0

[cc] Period elapsing after the male and female rats were put in the same cages. [dd] Average weights of 7-litter rats only. Figures in parentheses give average number of rats. [ee] Experiment terminated before all rats were weaned.

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(1.9% SC), 98% (53 of 54); and Diet 188 (9.5% SC), 97% (62 of 64). A 100% fertility was obtained in the fourth-generation rats from the control and both experimental groups. The body weights of the young were slightly higher in the experimental groups than in the control group; this would indicate that no interference with the lactation process had been occasioned by SC. The average weights of 3-day-old rats of all 4 generations were 6.9 g. for the control group, 7.0 g. for those receiving 1.9% SC, and 7.6 g. for those receiving 9.5% of SC in the diet. After 21 days, the comparable averages were 34.1, 35.4, and 37.0 g., respectively. Mortality from the 3rd to the 21st day was 15 for the control group, 5 for those which received 1.9%, and 2 for those which were fed 9.5% of SC.

In the 10-week growth tests, the averages of the mean weights of the 4 generations on the several dietary regimens showed little difference. The values for the male rats gave a grand average of 262.8, 261.1, and 255.9 g. for Diets 171, 187, and 188, respectively. In the case of the females, the corresponding grand averages were 172.7, 174.8, and 172.4 g., respectively. The mean weights of the rats after 10 weeks on both experimental diets containing SC were approximately identical, after 4 generations, with those of the first-generation animals on the same dietary regimen.

Growth Tests on Rats with Diets Containing Heated Fats Without or With Citric Acid Esters

Since it is known that some fats such as sardine oil become less completely digestible when subjected to a high temperature *(15)*, it was considered desirable to make tests on margarine fat heated without or with IC plus Vehicle or SC to determine whether a change in nutritional value may occur. In order to establish whether such a decrease occurs, growth tests were made on weanling rats using fats so treated as well as potato chips prepared by deep-fat frying in such fats. If the growth responses of the heated fats containing either of the citric acid esters tested were found to be inferior to that of the control heated fat, such an effect might be ascribed either to decomposition products of the citric acid esters or to a decomposition of the fat caused by heating in the presence of the citric acid esters. Data on these tests are summarized in Table 11.

No adverse effects on growth were noted in either male or female rats over 10-week periods when margarine fats heated at 205°C. continuously for 8 hours and used for deep-fat frying were incorporated in the diets, as compared with control tests in which unheated margarine fat was employed in similar dietary regimens. Moreover, when IC plus Vehicle or SC was added to the test oils prior to the prolonged heating procedure, and these oils were then subjected to deep-fat frying for the 8-hour period, no toxicity developed. This fact was demonstrated by identical growth curves of rats receiving diets containing the fat heated with additives as compared with those of rats receiving diets containing the heated fat without additives as well as the unheated fat without additives.

In addition, no differences in the growth of weanling rats were observed when they were fed diets containing potato chips fried in margarine fats with additives over that noted when the potato chips had been prepared in margarine fat without these citric acid esters. These results indicate

TABLE 11

The Increase in Weight of Weanling Rats over 10-Week Periods Which Received Diets Containing Unheated Margarine Fat (Control), Margarine Fat Heated at 205°C. for 8 Hours Without or With IC plus Vehicle or SC, and Potatoes Fried in Such Fats

Diet No.	Category	Test material in diet[ff]	Number of rats	Body weight		Gain in weight	Total food eaten	Total test substance eaten	Efficiency of diet[gg]
				Start	End				
	Experiments on male rats								
		%		g.	g.	g.	g.	g.	
70	Unheated fat (Control)	0	10	35.4	258.2	222.8	819	0	6.68
73	Heated fat	0	10	36.2	265.5	229.3	758	0	7.08
89	Heated fat plus IC plus Vehicle	0.06	10	37.3	262.3	225.0	748	0.45	7.04
74	Potato chips from 73	0	10	39.9	260.4	221.4	758	0	7.43
90	Potato chips from 89	0.044	9	36.3	263.0	226.4	789	0.35	7.04
189	Heated fat plus SC	0.86	10	38.0	267.5	229.5	776	6.67	6.93
190	Potato chips from 189	0.68	11	39.0	265.1	226.1	793	6.40	6.68
	Experiments on female rats								
70	Unheated fat (Control)	0	10	39.7	180.2	140.5	701	0	4.93
73	Heated fat	0	10	38.1	187.0	148.9	670	0	5.20
89	Heated fat plus IC plus Vehicle	0.06	9	38.0	193.7	155.7	680	0.41	5.37
74	Potato chips from 73	0	9	37.2	182.1	144.9	677	0	5.42
90	Potato chips from 89	0.044	9	39.2	185.7	146.5	689	0.31	5.22
189	Heated fat plus SC	0.86	9	38.9	180.8	141.9	649	5.58	5.12
190	Potato chips from 189	0.68	8	39.4	181.3	141.9	699	5.65	5.00

[ff] Based on content of citrate esters present in fat at start of heating. [gg] (G. gain in weight/calories consumed) × 100.

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The authors wish to
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73	Heated fat	0	10	38.1	187.0	148.9	701	0	4.93
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** Based on content of citrate esters present in fat at start of heating. ** (G. gain in weight/calories consumed) × 100.

that the heating of IC plus Vehicle or SC in margarine oil for 8 hours at 205°C. does not produce toxic decomposition products nor does it cause the oil to be adversely affected.

Acknowledgment

The authors wish to acknowledge the help of Dr. Wm. Baber, Mr. George Kryder, Mr. Lawrence Owens, and Mrs. Evelyn Savage during the course of the experiments.

SUMMARY AND CONCLUSIONS

The results of the present toxicological study of 2 new chemicals proposed for use as food additives may serve as an illustration of what constitutes adequate testing of new food chemicals. Mixtures of mono-, di-, and tri-alkyl esters of citric acid were evaluated for the safety of their use in stabilizing the flavor of oils and fats and foods containing such oils and fats.

Consideration was first given to an independent analysis on the part of the testing laboratory of the mixed esters of citric acid to check the claims made by the manufacturer that the products were actually what they were reputed to be. Because of the variations in composition of mixed esters, many different batches were employed in the current investigation; analyses were made of representative batches.

Consideration was then given to the possible hydrolytic products of the esters of citric acid, and evidence was cited from the literature showing that such hydrolytic products in themselves exhibit no toxicity. It was emphasized that the intact esters themselves must be subjected to exhaustive toxicological studies using 3 species, not more than 2 being rodents. LD_{50} values carefully determined on one species and checked with another, acute experiments on 3 species, longevity tests, multigeneration studies, and toxicological studies of the possible degradation products of the citric acid esters were all conducted. In addition, histopathological examinations of tissues obtained from sacrificed animals were made throughout the course of the investigation. When the major portion of the studies was completed, the data were presented to the Division of Pharmacology of the U. S. Food and Drug Administration for their criticism, and their suggestions were followed in carrying the toxicological investigation to completion.

More specifically, studies have been undertaken to determine whether 2 new flavor-stabilizing agents for fat products, namely isopropyl citrate esters, predominantly mono-isopropyl citrate (IC), the same plus mono- and diglycerides vehicle (IC plus Vehicle), and stearyl citrate esters, predominantly distearyl citrate (SC), possess any toxicity when measured by various appropriate indices.

The LD_{50} values for the 2 compounds studied were relatively high. That for IC plus Vehicle alone or dissolved in oil was approximately 19.0 g./kg. (7.2 g./kg. as IC), while the figure for SC exceeded 5.4 g./kg. in rats. No sex differences were noted.

There is no evidence from acute experiments on rats (6 weeks), rabbits (6 weeks), or dogs (11-12 weeks) of any toxicity as measured by growth, mortality, or pathological manifestations.

Longevity (chronic) tests were carried out on rats, for 2-year periods from weaning, on IC plus Vehicle at several levels (0.28, 0.56, and 2.80% of the diet) and on SC at 3 levels (0.5, 2.0, and 10.0% of the diet). No deleterious effects as compared with that of the controls were noted in any groups in so far as could be determined by growth rates, mortality, and histopathological examinations of tissues.

Another method for evaluation of possible chronic toxicity, namely multigeneration studies, likewise indicated that IC plus Vehicle, at a level of 2.8% of the diet, and SC, at levels of 1.9 and 9.5% of the diet, are completely innocuous. These conclusions are based upon fertility over 5 and 4 generations, lactation performance as judged by the 3-day and 21-day weights of the offspring, as well as by their survival for the nursing period, and by the rate of growth of rats of the several generations over a 10-week period following weaning.

Margarine fat containing 0.5% IC plus Vehicle or 7.5% of SC did not develop toxicity (as determined by rat feeding tests over 12 weeks) when subjected to deep-fat frying at 205°C. over an 8-hour period. Potato chips, fried in such fats, and which retained approximately 40% of the fat, were also entirely satisfactory for growth.

Histopathological examination of the tissues of the rats after 2 years, of rabbits after 6 weeks, and of dogs after 11 to 12 weeks on the test diets did not reveal any evidence of specific damage. In the case of rats and rabbits, the following tissues were subjected to histopathological examination: liver, kidney, gastrointestinal tract, spleen, heart, lung, pancreas, gonads, brain, and adrenals. Only the liver and kidneys were examined in the dog tests.

All tests for toxicity indicate that IC when given as IC plus Vehicle and SC are harmless at levels as high as 1.1 and 10% of the diet, respectively. The maximum level at which IC has been tested and proved to be harmless is more than 500 times that which would be ingested in margarine containing 0.02% of IC where this fat makes up 15% of the diet by weight. Similarly, the highest level at which SC has been tested and which has proved harmless is about 500 times greater than would be consumed when margarine containing 0.15% of SC makes up 15% of the diet by weight.

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TOXICOLOGICAL STUDIES ON ISOPROPYL AND STEARYL CITRATES[a,b]

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(Manuscript received January 6, 1951)

In view of the increased demand for margarine in recent years, a need has developed for new sources of edible vegetable oils for hydrogenation. Since the production of soybean oil has been markedly increased in the United States within the last decade, the margarine industry has naturally turned to this oil to supplement the supply of other vegetable oils. Thus, while 87 million pounds of soybean oil were used in 1941 for hydrogenation, out of a total of 257 million pounds of all vegetable oils, the quantity of this oil employed in a 12-month period, ending June 30, 1950, was reported as 233 million pounds out of a total of 701 million pounds used by the margarine industry. In other branches of the edible fat industries, there has also been an increased usage of soybean oil.

The chief difficulty in the use of soybean oil is its property of undergoing a so-called "flavor reversion." This change results in the development of an unpleasant taste. The reversion begins in both limpid and hydrogenated oils almost immediately after deodorization. According to Martin, Schepartz, and Daubert *(20)*, Schepartz and Daubert *(30)*, and Stapf and Daubert *(32)* this reaction is associated with the production of carbonyl compounds.

Citric acid is especially satisfactory as an anti-flavor-reversion agent. However, neither citric acid nor its salts dissolve in sufficient amounts in oil to be used conveniently for this purpose.

Vahlteich, Gooding, and Neal in rotating authorship *(8, 9, 10, 11, 22, 23, 24, 35, 36, 37, 38, 39)* have recently reported the synthesis and effectiveness of fat-soluble esters of citric acid in inhibiting the flavor-reversion of soybean oil, and in stabilizing the flavor of other fatty foods. The compounds were mixed mono-, di-, and tri-esters of citric acid, containing from 3 to 18 carbon atoms in the alcohol moiety. The mono-esters were the effective agents in stabilizing oil flavor while the di-esters, particularly the higher homologues, exhibited in addition emulsifying properties.

Before the above-mentioned esters of citric acid can enjoy any widespread application in foods, it is necessary to prove that they are not toxic.

Obviously, it is not possible to test in a thorough manner all of the esters of citric acid mentioned in the patents cited. The lowest and highest homologues in the series were subjected to exhaustive toxicological and metabolic studies. The lowest homologue selected was the mixture of

[a] Supported by a grant from The Best Foods, Inc. Some of these results were reported at the joint meeting of the Northern and Southern California Sections of the Institute of Food Technologists in February, 1949.

[b] Contribution No. 275 of the Department of Biochemistry and Nutrition.

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isopropyl citrate esters, predominantly the mono-ester, and the highest homologue selected was the mixture of stearyl citrate esters, predominantly the di-ester, having a melting point of 57 to 58°C. By the above it is not meant that the toxicity studies represent a blanket investigation of all homologues between the isopropyl and stearyl citrate esters. The basis of selection of the lowest homologue was because of its greatest effectiveness per unit weight in stabilizing the flavor of oils. The stearyl esters were chosen because these were far more readily soluble in oils than the lower members of the series. Indeed this same reasoning constitutes the bases for selecting these two types of esters in commercial application *(25)*.

As far as the hydrolysis products of these selected esters of citric acid are concerned, there is no danger of toxicity, even for a consumption of many times the levels required to preserve the oils. Both hydrolysis products of stearyl citrate esters are components of animal tissues, and are produced by normal metabolic processes. Not only are citrates widely distributed components of foods such as the citrus fruits and milk, but they are also intermediary products of carbohydrate metabolism in the so-called citric acid cycle *(14, 33)*. At the present time, the use of "citric acid and harmless citrates" is permitted in margarine without any specified limit, but only when used for the purpose of producing diacetyl *(6)*.

Stearyl alcohol has been found by Schoenheimer and Hilgetag *(31)* to occur in the feces of dogs; cetyl alcohol, however, was found to be a normal constituent of the feces of dogs, cats, and men. In fact, Stetten and Schoenheimer *(31)* have demonstrated that an interconversion of stearyl alcohol and stearic acid is a usual metabolic process. Moreover, stearyl stearate has been shown by Hilditch *(12)* to account for 2.5% of the total lipid in the head oil of the sperm whale.

Although isopropyl alcohol has toxicological properties at relatively high levels, no effects of this compound have been demonstrated which do not also result from the ingestion of ethyl alcohol *(16)*. Isopropanol has been shown to be approximately twice as toxic as is ethyl alcohol *(18)*. However, doses from 0.75 to 5.28 ml. of isopropanol per kg. body weight per day have been administered to rats without the development of any untoward symptoms. Administration of this alcohol over prolonged periods failed to show evidence of any delayed toxic effects *(16)*. It was likewise found that no deleterious effects on growth, pregnancy, or lactation resulted over several generations in rats given considerable quantities of isopropanol daily *(19)*.

The isopropyl citrates are dispersible in fat with some difficulty whereas the stearyl citrates are readily soluble in fat. For this reason, the first type of citric acid ester is introduced into an oil in a suitable vehicle. For this purpose, a mixture of mono- and diglycerides (1:1) of a vegetable oil is most desirable *(39)*.

The mixture of mono- and diglycerides which is used in conjunction with isopropyl citrate cannot be considered to be a substance possessing toxicity. Mono- and diglycerides occur physiologically in the digestion of fats *(7)*. Alpha-monopalmitin has been identified as a minor constituent of adrenal lipids *(29, 42)*, while Jones, Koch, Heath, and Munson *(13)* have recently reported the isolation of this monoglyceride from normal

hog pancreas. The satisfactory utilization of monoglycerid , in feeding experiments has been reported by several workers. Ames, O'Grady, Embree, and Harris *(1)* have recorded that the growth and reproduction in rats fed diets containing 15 or 25% of monoglycerides, over 3 generations, were just as satisfactory as was the case when the corresponding amounts of cottonseed oil were employed. Mattson and Beck *(21)* also obtained as good growth in rats when mono- and diglycerides were incorporated in the diet as when corresponding amounts of neutral fats were used. These results are in line with unpublished results obtained in our laboratory. They also confirm the earlier experiments of Braun and Shrewsbury *(2)*, who studied the nutritional value of monostearin. At the present time mono- and/or diglycerides of fat-forming fatty acids are permitted in margarine *(6)*.

Although the experimental data cited above indicate that the hydrolysis products of isopropyl citrate and stearyl citrate esters, as well as mono- and diglycerides, are in most cases natural products and are non-toxic at relatively high doses, it is still necessary to prove that the unhydrolyzed compounds do not possess specific toxicities not revealed in studies of the individual components. For this reason, the experiments reported here on rats, rabbits, and dogs were carried out. No previous toxicological investigations have been reported on isopropyl or stearyl citrate esters.

The test compounds used are mixtures of mono-, di-, and tri-alkyl esters of citric acid. In Table 1 are listed the typical compositions of the compounds investigated. From the acid value and saponification value, it is possible to estimate the ratio of mono- to di- to tri-ester for each of the types under study. It will be noted that, in the case of isopropyl citrates, the mixture is predominantly mono-isopropyl citrate and that in the case of the stearyl citrates the mixture is predominantly distearyl citrate.

In the preparation furnishing the isopropyl citrate esters, there is about 62% of the mixture of mono- and diglycerides. Analyses conducted in our laboratory for the alcohol moieties in the isopropyl and stearyl citrate esters checked very closely with the calculated values confirming the composition of the esters as given in Table 1. Analyses for citric acid gave values somewhat below the calculated values.

It will be noted in the footnotes to Table 1 that there are some variations in the ratio of mono- to di- to tri-ester in each category. It is pertinent to point out that in the present toxicological studies and in the metabolic studies reported elsewhere *(3)* many different batches of the mixed esters were employed (12 different batches in the case of the isopropyl citrate preparation and 11 in the case of the stearyl citrates). Analysis of the individual batches indicated that all samples fell in the range of composition indicated in the footnotes to Table 1.

For the purpose of convenience in referring hereinafter to the test materials employed in this study, the isopropyl citrate esters, predominantly mono-isopropyl citrate will be referred to as IC. Similarly, the stearyl citrate esters, predominantly distearyl citrate, will be referred to as SC. When the isopropyl citrate ester, predominantly mono-isopropyl citrate, is fed in the mono- and diglyceride vehicle, it will be referred to as IC plus Vehicle.

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TABLE 1

Typical Compositions of Compounds Investigated

Substance	Melting point, °C.	Acid value	Saponification value	Composition	Typical analyses[a]		
					Isopropyl alcohol[d]	Citric acid[e]	Stearyl alcohol[f]
					%	%	%
Isopropyl citrates in a vehicle consisting of mono- and diglycerides (1:1) of vegetable oil.	Liquid	160	370	27% mono-isopropyl citrate[g]	10.9	26.6	.....
				9% di-isopropyl citrate	(11.0)	(30.0)	.....
				2% tri-isopropyl citrate			
				62% mono- and diglycerides			
Stearyl citrates	57.8	88.6	248.1	12.5% monostearyl citrate[h]	.....	25.5	77.9
				75% distearyl citrate	.....	(28.3)	(76.8)
				12.5% tristearyl citrate			

[a] Figures in parentheses are calculated values.
[d] Isopropyl alcohol was determined after oxidation to acetone by refluxing with aqueous potassium dichromate and by precipitation with Deniges reagent *(40)*.
[e] Citric acid was determined on the aqueous extract after saponification by the method of Pucher, Wakeman, and Vickery *(28)* as modified by Perlman, Lardy, and Johnson *(27)*.
[f] Stearyl alcohol was determined as NSF by purification of the residue after saponification by filtration, drying, re-solution, and removal of the solvent.
[g] The 38% of isopropyl citrates is composed of about 65-80% mono-isopropyl citrates, 15-30% of di-isopropyl citrates and 5-10% tri-isopropyl citrate. Typical acid and saponification values are 410 and 690 respectively.
[h] The stearyl citrates are composed of about 10-15% monostearyl citrate, 70-80% distearyl citrates, and 10-15% tristearyl citrates.

The most important factors which determine the behavior of a substance in the body are its digestibility and absorption. If a substance is non-absorbable and at the same time non-irritating to the gastrointestinal tract, there is little possibility that it will exhibit any toxic effects. Data on the absorption and digestibility of isopropyl citrates, stearyl alcohol, and stearyl citrates have been presented in another communication *(3)*.

It was found that IC plus Vehicle in large amounts did not lower the high digestibility of margarine fat when fed to the rat or dog. On the other hand, when SC was fed at very high levels to the rat, the digestibility of the margarine fat was somewhat depressed. However, when the SC was given at a level of 3 times the maximal proposed level *(25)*, assuming that all the fat in the diet was so treated, the digestibility of the margarine fat was not appreciably altered *(3)*. In the case of the dog, no significant depression in digestibility of the margarine fat occurred when the level of SC in the fat was fully 80 times the maximal proposed level.

EXPERIMENTAL

In order to check for any possible toxicological effects of isopropyl citrate esters, predominantly mono isopropyl citrate (IC), the same plus the mono- and diglyceride vehicle (IC plus Vehicle), and of stearyl citrate esters, predominantly distearyl citrate, (SC), tests have been carried out on rats, rabbits, and dogs. The use of the several species in such studies is in line with the suggestions of Lehman, Lang, Woodard, Draize, Fitzhugh, and Nelson *(17)* that at least 2 species (including one which is a non-rodent) be employed.

All the rats used in the several types of experiments were from our stock colony. LD_{50} studies were conducted on IC, IC plus Vehicle, and on SC.

Acute tests were made on male and female rats at 3 levels for each test substance for a 6-week period. Control experiments in which no test substance was included in the diets were carried out in each series. In most cases, 10 rats of each sex were used per group.

The third type of test (chronic) employed with the rats was essentially a longevity experiment carried out over a period of 2 years with the same distribution in groups as in the acute tests. In both the acute and the chronic tests, the animals were sacrificed at the end of the experiment, and tissues were obtained for histopathological examination.

Another procedure used here for the dectection of any possible chronic or cumulative toxicity includes multigeneration studies. These involved raising the first generation of rats from weaning on the several test diets, and on a control diet, and continuing them on these regimens during the breeding and the lactation periods. When the young were weaned, the parents were discarded and the second generation was continued on the same diets which the parents had received. A similar routine was employed for succeeding generations through the weaning of the sixth generation, in the IC plus Vehicle tests, and of the fifth generation in the tests with SC.

In still another approach, additional groups of rats were used to ascertain whether these esters of citric acid in oils develop a toxicity when heated at 205°C. (a temperature used in deep frying) over a prolonged period (8 hrs.). The oils previously heated with or without the citric acid ester, as well as potato chips prepared by frying in such oils, were incorporated in diets which were then tested by growth studies on weanling rats over 11-week periods.

In the complete series of tests reported here (not including the absorption and digestibility studies), a total of 2398 rats was used.

Rabbits and dogs were employed for acute toxicity studies on the citric acid esters. The rabbit tests were continued for 6 weeks, while the experiments on dogs lasted for a 12-week period. A histopathological examination was made of the tissues of all of the test animals at the conclusion of the experimental period. In addition, massive single doses of IC plus Vehicle or of SC were administered to dogs. A total of 57 rabbits and 24 dogs was used in the toxicity tests.

The LD_{50} tests were carried out by the administration of the test substance by stomach tube to the fasted animals. In the rat tests, studies were made on IC, an equivalent isopropanol-citric acid mixture as control, IC plus Vehicle alone and in cottonseed oil, cottonseed oil alone as a control, and SC in cottonseed oil. The tests were considered positive if the animals died within periods of 4 to 6 days; this interval was chosen since, at that time, practically all the animals remaining alive were again gaining weight and in good nutritional condition. The dog experiments were carried out in essentially the same manner but because of necessity the number of test animals was restricted.

The diets used in the rat and dog experiments were made up of purified foodstuffs. The compositions of the various diets used are listed in Tables 2 and 3.

The rats used on the LD_{50} tests had been maintained on our usual stock diet. They were fasted for 18 hours prior to the administration of the test substance by stomach tube.

Rabbits were fed throughout the tests on commercial rabbit pellets.[a] IC plus Vehicle was mixed with the rabbit pellets in amounts of 0, 1.9, 3.8, 7.5, or 15.0% (0.0, 0.72, 1.4, 2.8, or 5.7% IC, respectively). In the tests with SC, the pellets were mixed with 2 or with 11% of the melted substance. The diets fed the dogs in the 3-month acute tests are patterned after those described by Cowgill *(4)*. Table 3 gives the composition of these diets.

The IC, IC plus Vehicle, and SC employed in the tests reported here were commercial products supplied by The Best Foods, Inc. The identity of the products was confirmed by our analysis. Hemoglobin determinations were made on the dogs by the acid hematin method.

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[a]Albers Rabbit Family Ration.

TABLE 2

The Composition of Diets Used in the Rat Tests

Dietary component	Acute tests					Chronic tests			Multigeneration tests				Heated oil tests			
	70	80	81	82	83	84	85	86	71	72	87	88	73	89	74	90
Isopropyl citrate esters, predominantly mono-isopropyl plus mono- and diglyceride vehicle (IC plus Vehicle)																
	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
Casein	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0
Sucrose	55.5	54.9	54.3	53.0	50.5	55.5	55.5	55.5	50.5	53.5	50.5	53.5	55.5	55.5	44.0	44.0
Whole margarine[i]	12.50	11.42	10.34	8.00	3.50	12.22	11.94	9.70	12.5	12.5	9.7	9.7	....	....	....	....
Margarine fat	....	....	....	....	....	....	....	....	....	....	....	....	11.5[j]	11.44[j]	....	....
Fat-soluble vitamin mixture[k]	....	....	....	....	....	....	....	....	....	....	....	....	1.0	1.0	1.0	1.0
Yeast[l]	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0
Salt mixture[m]	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Liver powder[n]	....	....	....	....	....	....	....	....	5.0[o]	2.0[p]	5.0[o]	2.0[p]	....	....	....	....
Potato chips:																
Fried in margarine fat	....	....	....	....	....	....	....	....	....	....	....	....	....	....	23.0	....
Fried in margarine fat containing IC plus Vehicle (0.5%)	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	23.0
IC plus Vehicle	....	1.68	3.36	7.00	14.00	0.28	0.58	2.80	....	....	2.8	2.8	....	0.06	....	....
IC content	....	0.64	1.3	2.7	5.3	0.11	0.21	1.1	....	....	1.1	1.1	....	0.02	....	....
Stearyl citrate, predominantly distearyl citrate (SC)																
Dietary component	170	180	181	182	183	184	185	186	171		187	188	73	189	74	190
	%	%	%	%	%	%	%	%	%		%	%	%	%	%	%
Casein	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	19.0		19.0	19.0	20.0	20.0	20.0	20.0
Sucrose	55.5	54.2	53.0	50.5	45.5	55.0	53.5	45.5	51.8		51.0	43.3	55.5	55.5	44.0	44.0
Margarine fat	11.5	11.5	11.5	11.5	11.5	11.5	11.5	11.5	11.0		9.9	10.0	11.5[j]	10.64	....	....
Fat-soluble vitamin mixture[k]	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0		1.0	1.0	1.0	1.0	1.0	1.0
Yeast[l]	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	7.6		7.6	7.6	8.0	8.0	8.0	8.0
Salt mixture[m]	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.8		4.8	4.8	4.0	4.0	4.0	4.0
Liver powder[n, o]	....	....	....	....	....	....	....	....	4.8		4.8	4.8	....	....	....	....
Potato chips:																
Fried in margarine fat	....	....	....	....	....	....	....	....	....	....	....	....	....	....	23.0	....
Fried in margarine fat containing SC (7.5%)	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....	23.0
SC	....	1.3	2.5	5.0	10.00	0.5	2.0	10.0	....		1.9	9.5	....	0.86	....	....

[i] 8 I U of vitamin D (as viosterol) added per g. food. [j] Heated for 8 hours at 205°C. [k] Composition was as follows: α-tocopherol, 0.5 g.; carotene (General Biochemicals, Inc.), 20 mg.; vitamin D_2 (crystalline Winthrop), 0.5 mg.; cottonseed oil to make a total weight of 100 g. [l] Anheuser-Busch, Strain G. [m] Osborne-Mendel salt mixture (*26*). [n] Wilson Laboratories. [o] 1:20 concentrate. [p] 1:50 concentrate.

TABLE 3

Composition of the Diets Used in the Experiments on Dogs

Food component	Composition of diet		
	D-1	D-2	D-3
	%	%	%
Commercial casein	20.0	20.0	20.0
Sucrose	35.0	35.0	35.0
Dextrin	12.2	12.2	12.2
Margarine oil[a]	18.3	18.24	15.3
Salt mixture (Reference 41)	2.0	2.0	2.0
Cellu flour[b]	2.5	2.5	2.5
Cod-liver oil (U.S.P.)	5.0	5.0	5.0
Dried brewers' yeast[c]	5.0	5.0	5.0
IC plus Vehicle	....	0.06	....
SC	....	....	3.0

[a] Without additives. [b] Chicago Dietetics Supply House. [c] Anheuser-Busch (Strain G).

RESULTS AND DISCUSSION

LD_{50} Studies

The acute toxicity, as revealed by the determination of the LD_{50} levels, has been ascertained for rats, and checked with dogs. The results of the tests on rats are summarized in Table 4.

The LD_{50} values of the several test materials as determined on rats are all quite high. The LD_{50} value with male rats for IC plus Vehicle, when given alone, exceeded 20.7 g./kg., while when it was administered in a ratio of one part of cottonseed oil to 2 parts of IC plus Vehicle, the value was greater than 17.2 g./kg. The corresponding figures for the female rats were 18.8 and 19.2 g./kg. This would indicate that supplementation of IC plus Vehicle with fat does not accentuate the LD_{50} figure.

When IC was administered in alcohol solution or in water, the toxicity was 2.8 to 3.7 g./kg. The slightly lower LD_{50} value for the females receiving IC in ethanol may be the reflection of the higher alcohol dose which was administered. In the tests with females where an aqueous solution was employed, the figure for LD_{50} approximated the value obtained for the males. The quantity of IC required to produce 50% mortality approximated that for its hydrolysis products. However, when IC is administered in a fat vehicle, more than double the amount of IC is required to produce a 50% mortality.

The LD_{50} level for SC exceeds 5.4 g./kg. in the case of both male and female rats. Since it was impossible to prepare a solution of SC, liquid at body temperature, in a higher concentration than 20%, the maximum dose (30 ml./kg.) which could be introduced into the stomachs of rats contained only 5.4 g./kg.

Twelve dogs were used to check the LD_{50} values obtained with rats. Massive single doses, viz. 12 g./kg. of IC plus Vehicle, 2.25 g./kg. of IC, and 5 g./kg. of SC, were not fatal to the dog. After a period of 2 days, the dogs were eating normally and showed no obvious ill effects from the dosage.

ents on Dogs

osition of diet	
D-2	D-3
%	%
20.0	20.0
35.0	35.0
12.2	12.2
18.24	15.3
2.0	2.0
2.5	2.5
5.0	5.0
5.0	5.0
0.06	....
....	3.0

user-Busch (Strain G).

ion of the LD_{50} levels, rs. The results of the

determined on rats are C plus Vehicle, when dministered in a ratio Vehi the value was s for the female rats t supplementation of $_{50}$ figure.

in water, the toxicity or the females receiv- er alcohol dose which aqueous solution was lue obtained for the % mortality approxi- en IC is administered s required to produce

ase of both male and tion of SC, liquid at , the maximum dose chs of rats contained

obtained with rats. e, 2.25 g./kg. of IC, a period of 2 days, s ill effects from the

TABLE 4

LD_{50} Tests on Rats

Substance tested	Sex of rats	Data on different levels of tests						Estimated LD_{50}[a]
IC plus Vehicle								*g./kg.*
Dosage (g./kg.)	M	2.1-20.7	.....	.....	.....	.....	.....	>20.7
Number of rats		6	.....	.....	.....	.....	.....	(>7.9)
Mortality (%)		0	.....	.....	.....	.....	.....	.....
Dosage (g./kg.)	F	2.7-15.5	17.7	21.0	23.6	27.0	.....	18.8
Number of rats		4	10	10	11	10	.....	(7.2)
Mortality (%)		0	30	90	89	100	.....	.....
IC plus Vehicle in cottonseed oil (2:1)								
Dosage (g./kg.)	M	0	11.1	14.1	16.1	17.2	.....	>17.2
Number of rats		10	9	10	9	9	.....	(>6.5)
Mortality (%)		0	11	20	22	33	.....	.....
Dosage (g./kg.)	F	0	12.0-13.4	15.8	17.9	19.2	22.2	19.2
Number of rats		10	20	10	10	10	10	(7.3)
Mortality (%)		0	0	20	10	50	50	.....
IC								
75% in 10% ethanol								
Dosage (g./kg.)	M	2.1	2.7	3.3	3.9	.....	.....	3.7
Number of rats		10	10	10	10	.....	.....	.....
Mortality (%)		0	20	10	70	.....	.....	.....
15% in 10% ethanol								
Dosage (g./kg.)	F	2.1	2.7	3.3	3.9	.....	.....	2.8
Number of rats		10	10	10	10	.....	.....	.....
Mortality (%)		10	40	90	100	.....	.....	.....
75% in water								
Dosage (g./kg.)	F	2.25	3.0	3.75	4.5	5.25	5.25[w]	3.6
Number of rats		10	10	10	10	10	10	.....
Mortality (%)		0	10	60	70	100	90	.....
Isopropyl alcohol plus citric-acid in ethanol								
Dosage (g./kg.)	F	3.9[v]	3.9	5.25[v]	5.25[v]	.....	.....	4.5
Number of rats		10	9	10	10	.....	.....	.....
Mortality (%)		0	22	90	100	.....	.....	.....
SC (20% in cottonseed oil)								
Dosage (g./kg.)	M	0	0.9-1.8	2.7	3.6	4.5	5.4	>5.4
Sol. vol. (ml./kg.)		30	5-10	15	20	25	30	.....
Number of rats		10	4	2	12	13	10	.....
Mortality (%)		0	0	0	8	15	10	.....
Number of rats	F	10	4	2	12	13	10	>5.4
Mortality (%)		0	0	0	0	0	0	.....

[a] Figures in parentheses represent IC content of mixtures. [v] Male rats. [w] In water.

Short-Term (Acute) Tests

Experiments on Rats. Tests were made on IC plus Vehicle or SC when incorporated in the commercial margarine fat. Table 5 summarizes these data. No animals maintained on any of the diets died during the course of the tests.

There is no evidence, on the basis of the acute tests on adult rats, that either IC plus Vehicle or SC possesses any degree of toxicity. Thus, adult

TABLE 5

The Change in the Weight of Adult Rats Receiving Control Diets Alone or Similar Diets to Which Several Levels of IC plus Vehicle or SC Were Added

Diet no.	Test material in diet	Experiments on male rats						Experiments on female rats					
		Number	Body weight		Increase in weight	Total food eaten	Total test substance eaten	Number	Body weight		Increase in weight	Total food eaten	Total test substance eaten
			Start	End					Start	End			
Isopropyl citrate esters, predominantly mono-isopropyl citrate, plus mono- and diglyceride vehicle (IC plus Vehicle)													
	%		g.	g.	g.	g.	g.		g.	g.	g.	g.	g.
70	0.0	10	336	386	50.2±2.7	625	0	10	221	247	26.5±3.5	496	0
80	1.68(0.64)*	10	326	364	38.3±5.3	568	9.54	10	208	230	21.8±4.6	447	7.51
81	3.36(1.8)	10	324	368	44.8±4.9	588	19.8	9	214	231	17.3±5.1	450	15.1
82	7.00(2.7)	9	331	373	42.0±6.7	597	41.8	10	210	226	15.5±3.5	437	30.6
83	14.00(5.3)	10	329	372	42.6±4.1	615	86.1	10	208	223	15.0±7.8	432	60.5
Stearyl citrate esters, predominantly distearyl citrate (SC)													
170	0	10	203	271	67.9±5.9	442	0	10	202	230	27.6±3.4	390	0
180	1.3	10	206	302	96.0±7.9	514	6.68	10	202	230	28.4±3.6	422	5.48
181	2.5	10	209	292	82.7±7.5	494	12.3	10	206	235	29.2±3.8	406	10.1
182	5.0	10	205	296	90.8±7.8	542	27.1	10	196	220	25.0±5.5	447	22.3
183	10.0	10	208	304	95.4±6.9	555	55.5	10	193	220	27.2±5.2	445	44.5

* IC content of IC plus Vehicle in parentheses.

male rats receiving IC plu this ester over a 6-week P female rats ingested an a per rat per day) without respond to a daily intake rat per day in the 2 sexes.

The results of the acute plus Vehicle. The average the highest dosage was 55.5 by the females was 44.5 g.

The slight average diffe between the control animal doses of IC plus Vehicle a on adult rats of about 6 mo parallelism with dosage. Lik ently better growth respon compared to the responses

Experiments on Rabbits. on rabbits receiving the IC animals were fed on the bas was administered daily to period. In the second and th and in all the tests SC with the crushed rabbit pell Table 6 shows the average r

The Change in Weight of Rabbits Levels of IC plus Vehicle or SC Mixed

Group No.	Series	Test material in diet	Number	Sta
				IC pl
1	I	% 0	2	g. 201
2	I	2.2	3	209
3	I	4.4	2	230
4	I	9.2	1	210
				IC plus
5	III[7]	0	8	250
6	II	1.9	1	20
7	II	3.3	2	20
8	II	7.5	2	25
9	II	15.0	5	216
10	III[7]	11.3	8	242
11	III	22.5	8	244
				S
12	III	2	8	253
13	III	10	8	251

[7] One rabbit died during e of

male rats receiving IC plus Vehicle at the highest level ingested 86 g. of this ester over a 6-week period (more than 2 g. per rat per day), and female rats ingested an average of 60 g. over the same period (1.42 g. per rat per day) without any demonstrable harmful effects. These correspond to a daily intake of 0.78 g. and 0.54 g. respectively of IC per rat per day in the 2 sexes.

The results of the acute tests on SC are quite similar to those on IC plus Vehicle. The average level of SC taken in by the male rats receiving the highest dosage was 55.5 g. (1.32 g. per rat per day) and that ingested by the females was 44.5 g. (1.06 g. per rat per day).

The slight average differences noted in the increases in body weight between the control animals and those receiving progressively increasing doses of IC plus Vehicle are not significant. These tests were conducted on adult rats of about 6 months of age and the growth responses show no parallelism with dosage. Likewise, no significance is attached to the apparently better growth responses of the adult animals receiving SC when compared to the responses of the control rats.

Experiments on Rabbits. Two types of experiments were carried out on rabbits receiving the IC plus Vehicle. In the first series of tests, the animals were fed on the basal diet (rabbit pellets) and the test substance was administered daily to the animals by stomach tube over the 6-week period. In the second and third series of experiments with IC plus Vehicle, and in all the tests with SC, the substances under examination were mixed with the crushed rabbit pellets before this material was fed to the rabbits. Table 6 shows the average results of these tests.

TABLE 6

The Change in Weight of Rabbits Receiving Control Diets or Those Containing Several Levels of IC plus Vehicle by Stomach Tube (Series I), or IC plus Vehicle or SC Mixed with Diet (Series II and III)

Group No.	Series	Test material in diet	Number	Body weight			Average gain in weight	Average total food eaten	Average test material given
				Start	3 weeks	6 weeks			
IC plus Vehicle by stomach tube									
		%		g.	g.	g.	g.	g.	g.
1	I	0	2	2010	2204	2770	760	Ad libitum	0
2	I	2.2	3	2098	2407	2899	801	food intake	120
3	I	4.4	2	2300	2598	2885	585	not record-	240
4	I	9.2	1	2104	2380	2780	676	ed.	500
IC plus Vehicle incorporated in diet									
5	III[7]	0	8	2503	2821	3263	760	5413	0
6	II	1.9	1	2092	2402	2735	641	5411	113
7	II	3.8	2	2026	2456	2985	959	5399	205
8	II	7.5	2	2550	2632	2902	352	5010	376
9	II	15.0	5	2166	2556	2927	761	5366	805
10	III[7]	11.3	8	2427	2952	3276	849	5175	585
11	III	22.5	8	2442	2702	3039	597	3803	856
SC incorporated in diet									
12	III	2	8	2532	2729	3171	640	4763	95.3
13	III	10	8	2516	2599	2950	434	4428	442.8

[7] One rabbit died during course of test.

Only 2 deaths occurred among the 57 rabbits during the course of the test. One of these was in Group 5 (control) and the second was in Group 10 (receiving 11.3% of the composite, IC plus Vehicle). The average intake of IC plus Vehicle of 4 groups of rabbits (22 animals) exceeded 500 g. during the 6-week period. The maximum consumption for the period was by one rabbit in Group 11. The total amounted to 1045 g., or 24.9 g. daily; this approximates a daily intake of IC plus Vehicle of 1% of the body weight.

The intake of the SC was somewhat lower, averaging 443 g. for the group on the higher dosage. The highest consumption in this group was 513 g. of the test substance for the period, or 12.2 g. per rabbit per day.

The average gain in weight for the control rabbits was 760 g. for the 6-week period. Several groups receiving IC plus Vehicle (Groups 2, 7, 9, 10) equalled or exceeded this figure. In both groups receiving SC, the average gain in weight was somewhat less. All rabbits receiving SC showed excellent increase in weight, except one animal which lost weight during the early part of the test when it failed to eat; this rabbit began to regain its weight during the last week of the test. It had returned to approximately its starting weight at the termination of the experiment.

A histopathological examination was made on 311 sections of 284 organs from 24 rabbits by one of us (H.R.F.). These included sections of the liver, kidney, heart, brain, lung, spleen, stomach, small intestine, large intestine, pancreas, adrenal, and testicle of the 8 rabbits receiving the highest dose of IC plus Vehicle (22.5%), of the 8 animals given higher dose of SC (10%), and of the 8 controls (Group 5) in Series III. These were all males. There was no obvious morphological evidence of disease in the tissues of any group given diets in the categories listed.

Experiments on Dogs. Acute tests were made on 12 dogs over 11 to 12-week periods. Three groups of 4 each were used. The dietary regimens included a basal diet (control group), a similar diet including 0.06% IC plus Vehicle, and a third to which 3.0% of SC was added. The dogs used consisted of a litter of 6 cocker puppies, which were distributed equally between the groups, and 6 adult mongrels of unknown nutritional history. The adults were devocalized and dewormed before the test was started, while the puppies were devocalized during the second week of the tests. Because of the uniformity in origin and dietary background of the puppies, greater significance should be attached to weight changes of these animals.

All dogs received measured amounts of food once daily. In the case of the adult dogs, the amounts were first based on the quantities necessary to establish an optimum body weight, as calculated from Cowgill's formula *(5)*. When this weight was attained, they were placed on a maintenance diet. In the case of the puppies, the diet was adjusted to give a steady rate of growth. Water was given ad libitum throughout. The data on these tests are included in Table 7.

There is no evidence of toxicity of IC plus Vehicle or SC when fed to dogs over a 12-week period. IC plus Vehicle was fed at a level of 0.25% of the fat, which is 10 times the level proposed for its use in margarine (0.02% of the whole margarine or 0.025% of the margarine fat). SC was fed at a level of 14.8% of the fat, which is 80 times the maximum level to

TABLE 7

The Change in Weight of Dogs Receiving a Control Diet or Diets Containing IC plus Vehicle or SC

Dog No.	Type of dog	Sex	Body weight: Start	Body weight: After 4 weeks	Body weight: After 8 weeks	Body weight: After 12 weeks	Gain in weight	Total food eaten	Total test substance eaten	Hemoglobin at end of test
			Control group (Diet D-1)							
			kg.	*kg.*	*kg.*	*kg.*	*kg.*	*g.*	*g.*	%
1	Cocker pup	M	2.3	3.2	3.5	3.6	1.3	7990	.....	12.9
2	Cocker pup	F	2.0	2.8	3.2	3.2	1.2	7927	.....	14.2
3	Mongrel adult	M	15.9	18.2	18.5	18.8*	2.9	27230	.....	15.9
4	Mongrel adult	M	13.5	15.4	15.9	16.3	2.8	16870	.....	17.6
Average**			2.15	3.00	3.35	3.40	1.25	7958	.....	.....
			Group receiving IC plus Vehicle (Diet D-2)							
5	Cocker pup	F	2.3	3.3	3.7	3.8	1.5	7918	4.75	12.6
6	Cocker pup	F	2.5	3.1	3.6	3.8	1.3	7663	4.60	14.0
7	Mongrel adult	M	10.9	12.8	14.8	15.0*	4.1	23330	13.98	14.3
8	Mongrel adult	F	5.0	6.4	6.8	7.4	2.4	17290	10.37	18.6
Average**			2.40	3.20	3.65	3.80	1.40	7790	4.68	.....
			Group receiving SC (Diet D-3)							
9	Cocker pup	F	2.1	3.2	3.7	3.8	1.7	7683	230.5	12.9
10	Cocker pup	F	1.8	2.6	3.2	3.3	1.5	7887	236.6	12.8
11	Cocker adult	M	12.7	15.5	17.1	17.3*	4.6	19320	579.6	13.6
12	Mongrel adult	M	5.2	4.8	5.0	5.7	0.5	7990	239.7	14.0
Average**			1.95	2.90	3.45	3.55	1.60	7785	233.6	.....

* Weight at 11 weeks. ** Average of litter mate cocker puppies only.

be used in margarine (0.15% of the whole margarine or 0.19% of margarine fat).

The amount of food consumed in the 3 groups was comparable. Actually, in the case of the puppies, the gain in weight of the experimental groups somewhat exceeded that of the control group, although the former animals consumed somewhat less food. The same pattern was followed by the adult dogs. Hemoglobin levels determined before the dogs were sacrificed were practically identical for the several groups. A histopathological examination of the livers and kidneys of the dogs failed to reveal any evidence of pathological manifestations attributable to the test substance. Whatever divergencies from normal occurred were distributed equally among the several groups.

Long-Term (Chronic) Tests on Rats

Longevity Tests. In order to determine whether the materials tested may exhibit a toxicological action when their administration is continued over a prolonged period, chronic tests were carried out on rats for 2 years. The tests were discontinued at the end of this interval, so that the tissues could be obtained for pathological examination from animals which had remained alive until the termination of the experiment. Data on the body weights of the rats in the various series of tests at representative periods during the experiment, as well as on their survival, are included in Table 8.

With the exception of the control group in the distearyl citrate tests, 55% or more of the rats were still alive after receiving the various diets for a period of 2 years. When allowances are made for accidental deaths, the percentage survival in the tests with IC plus Vehicle were 59, 82, and 58% for the groups receiving the low, intermediate, or high level of test substance, with the value of 63% for the control group. In the experiments in which SC was added to the diets, the survivals amounted to 66, 65, and 55% for the groups receiving the progressively increasing proportions of the test compound, with an average of 44% for the corresponding control group.

No marked variations in weight between the experimental and the control groups were noted during the 2-year period. However, after 104 weeks, the average of the means of the weights of males and females still surviving in the IC plus Vehicle tests were as follows: Diet 70 (control), 414 g.; Diet 84 (0.28%), 400 g.; Diet 85 (0.56%), 425 g.; and Diet 86 (2.8%), 404 g. In the tests with SC, the corresponding average weights were: Diet 170 (control), 380 g.; Diet 184 (0.5%), 486 g.; Diet 185 (2.0%), 413 g.; and Diet 186 (10%), 388 g.

Histopathological sections of liver, kidney, heart, brain, lung, spleen, stomach, small intestine, large intestine, pancreas, adrenal, and testicle or ovary were examined. In the case of the rats receiving IC plus Vehicle at a 2.8% level, it is reported as follows: "It is my opinion that no specific pathological lesions developed which could be attributed to the product fed."[bb] An examination of the same tissues of a series of rats which had

[bb] Report of Professor Ernest M. Hall, Department of Pathology, School of Medicine, University of Southern California.

TABLE 8

The Body Weights and Survivals of Rats Receiving Control Diets or Diets Containing Several Levels of IC plus Vehicle or SC

Category	IC plus Vehicle tests				SC tests			
	Diet 70	Diet 84	Diet 85	Diet 86	Diet 170	Diet 184	Diet 185	Diet 186
Test substance in diet, %	0	0.28	0.56	2.80	0	0.5	2.0	10.0
IC content	0	0.11	0.21	1.06	....	....	....	....
Male rats								
Number in group	10	10	10	10	10	9	10	10
Body weight:								
Start, g.	35	36	36	36	35	33	34	32
After 32 weeks, g.	339	362	346	324	361	361	338	328
After 64 weeks, g.	421	471	452	419	423	475	423	423
After 96 weeks, g.	488	546	507	467	471	597	488	474
After 104 weeks, g.	520	495	543	461	454	610	524	482
Rats alive at end	7	5	5	4	3	4	6	5
Female rats								
Number in group	10	10	10	10	10	10	10	10
Body weight:								
Start, g.	40	38	39	39	30	32	32	28
After 32 weeks, g.	228	230	216	222	220	222	208	217
After 64 weeks, g.	287	268	263	255	267	282	272	259
After 96 weeks, g.	308	296	316	328	297	359	294	309
After 104 weeks, g.	309	304	307	348	305	363	302	294
Rats alive at end	5	5	9	7	5	8	7	6
Survival of all rats								
Uncorrected, %	60	50	70	55	40	60	65	55
Corrected for those killed accidentally, %	63	59	82	58	44	66	65	55

received distearyl citrate at a 10% level for 2 years was made by one of us (H.R.F.). Although most of the rats exhibited some diseased tissues characteristic of old age, no specific changes traceable to the diet could be noted. Metastatic calcification and tumor formation were noted in certain tissues examined; however, they are not considered to have special significance, inasmuch as they were noted in the tissues of both test and control rats.

Multigeneration Tests. The most sensitive method for the detection of dietary deficiencies or toxicity involves subjecting several generations of the animals to the test diets. All the factors of stress are brought to bear on the animal in such tests, namely growth, pregnancy, and lactation. Diets which are entirely satisfactory for maintenance, or even for growth, over a single generation may fail to be sufficient for reproduction; moreover, a dietary regimen which serves adequately for both growth and reproduction may be unsatisfactory for lactation. The latter stress offers the most severe test of the nutritional value of a diet.

In the multigeneration tests, weanling male and female rats (Generation 1) whose parents had been on the laboratory diet were raised on the test diets and were bred after 10 weeks (at 13 weeks of age). These rats were continued on the test diets during the subsequent pregnancy and lactation. Young from the first generation (Generation 2) were continued after weaning on the same test diets which their parents had received. At maturity the second-generation rats were bred and a routine similar to the above was repeated. In the case of the rats receiving IC plus Vehicle, the experiment was continued through the weaning of the sixth generation; in the tests with SC, the experiment was terminated when the fifth-generation rats were weaned. Table 9 summarizes the data on the growth of the successive generations, while Table 10 records the information to be gained from the pregnancy and lactation performances.

The multigeneration tests indicate that IC plus Vehicle is completely harmless when incorporated in the diet at a level of 2.8% (equivalent to 1.1% of IC). The rats remained fertile throughout the 5 generations. The total litters cast in the test group comprised 91% of the females bred (59 of 65); this figure was identical with that of the control group (90%, 65 of 72). The body weights of the progeny were likewise similar in the control and experimental groups. At the age of 3 days, the average weights were 7.6 and 7.5 g., while at the age of 21 days the mean values were found to be 34.5 and 35.1 g., respectively.

The results of the growth tests carried on from weaning for the successive 10-week period give added confirmation of the non-toxicity of IC plus Vehicle at the 2.8% level. The average 10-week weight of the fifth generation male and female rats on the test diet (254.7 g., 178.3 g.) not only exceeded the average of the first-generation rats on this test diet for the 10-week period (238.7 g., 176.6 g.) but it was also higher than that of the fifth generation of the control rats (223.4 g., 151.2 g.).

A similar analysis of the data indicates that SC exhibits no toxicity over 4 generations when fed at a level of 1.9% or 9.5% of the diet. The fertility remained high in all groups. The percentages of successful pregnancies were as follows: Diet 171 (control), 93% (55 of 59); Diet 187

TABLE 9

Body Weights of Successive Generations of Rats Receiving Control Diets or Those Containing IC plus Vehicle or SC

Diet No.	Test substance in diet	Generation	Male rats				Female rats			
			Number	Body weights			Number	Body weights		
				Start	After 5 weeks	After 10 weeks		Start	After 5 weeks	After 10 weeks
IC plus Vehicle tests										
	%			g.	g.	g.		g.	g.	g.
71	0	1	10	30.1	158.6	246.9	15	30.5	123.6	175.3
		2	9	38.6	168.5	281.5	15	37.8	137.9	187.3
		3	10	36.5	151.1	243.0	16	34.5	113.2	157.1
		4	14	38.8	153.2	224.3	22	37.4	123.0	159.4
		5	13	37.5	143.8	223.4	26	35.9	113.8	151.2
87	2.8	1	11	30.4	155.2	238.7	15	30.3	133.0	176.6
		2	11	38.5	157.7	255.0	12	34.6	132.8	182.1
		3	17	35.0	134.7	227.5	16	34.5	121.2	174.7
		4	12	39.5	164.4	240.0	18	37.9	124.5	160.4
		5	17	38.7	178.1	254.7	21	37.6	136.3	178.3
SC tests										
171	0	1	5	37.8	164.0	265.0	10	37.3	135.7	182.5
		2	14	34.0	156.6	243.6	21	35.7	125.2	166.3
		3	16	37.4	168.2	253.5	19	35.8	122.7	163.1
		4	18	36.9	161.7	289.3	22	36.5	126.1	179.0
187	1.9	1	5	38.2	167.4	273.0	10	37.3	132.8	177.5
		2	19	34.9	150.1	245.3	16	33.9	122.0	171.1
		3	14	37.1	175.2	257.8	20	35.5	131.7	172.9
		4	10	40.3	172.9	268.4	16	38.6	129.1	177.8
188	9.5	1	4	36.5	160.5	267.3	10	37.6	131.5	177.4
		2	19	36.6	153.7	246.0	26	32.5	125.3	165.4
		3	10	33.2	162.2	237.1	19	32.7	124.7	166.2
		4	13	43.8	174.9	273.3	19	39.5	132.0	178.8

TABLE 10

Pregnancy and Lactation Response of Successive Generations of Rats Receiving Control Diets or Diets Containing IC plus Vehicle or SC

Diet No.	Test substance added	Parents: Generation	Parents: Number of rats bred	Parents: Number of litters cast	Parents: Average time[cc]	Litter: Generation	Litter: Average number	Litter: Body weight 3 days	Litter: Body weight 21 days[dd]	Litter: Total rats weaned	Litter: Died between 3rd and 21st day
IC plus Vehicle tests											
	%				*days*			*g.*	*g.*		
71	0	1	15	13	31.0	2	8.2	7.6	38.3 (49)	70	0
		2	10	10	43.2	3	6.2	7.3	28.1 (14)	33	4
		3	10	10	35.4	4	6.9	8.0	38.6 (28)	56	3
		4	22	17	34.9	5	6.4	7.7	37.0 (21)	55	1
		5	25	25	36.1	6	5.8	7.3	30.4 (28)	103+[ee]	1
87	2.8	1	12	12	28.8	2	9.9	7.5	35.8 (77)	77	0
		2	8	8	31.4	3	8.3	6.9	34.5 (35)	35	0
		3	7	7	38.2	4	6.7	7.7	35.2 (14)	46	0
		4	17	17	29.6	5	7.9	8.2	34.3 (91)	98	0
		5	21	15	42.2	6	6.3	7.2	35.9 (28)	56+[ee]	0
SC tests											
171	0	1	10	9	28.0	2	9.4	6.9	34.0 (35)	35	0
		2	14	13	27.3	3	8.3	6.6	34.3 (35)	60	3
		3	19	17	27.7	4	7.7	7.2	33.6 (35)	65	5
		4	16	16	26.6	5	7.1	6.9	34.5 (63)	72	7
187	1.9	1	10	10	26.8	2	6.3	6.7	34.6 (21)	41	0
		2	15	15	26.2	3	6.8	7.4	33.5 (42)	58	0
		3	15	14	24.4	4	8.1	7.0	36.9 (56)	83	3
		4	14	14	31.0	5	8.4	6.8	36.7 (56)	71	2
188	9.5	1	11	11	30.2	2	7.6	7.0	34.5 (43)	61	2
		2	23	23	26.2	3	8.5	7.2	35.5 (105)	117	0
		3	16	14	26.5	4	8.5	8.5	41.0 (56)	67	0
		4	14	14	27.4	5	9.4	7.6	37.1 (84)	94	0

[cc] Period elapsing after the male and female rats were put in the same cages. [dd] Average weights of 7-litter rats only. Figures in parentheses give average number of rats. [ee] Experiment terminated before all rats were weaned.

(1.9% SC), 98% (53 of 5
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Growth Tests on With

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(1.9% SC), 98% (53 of 54); and Diet 188 (9.5% SC), 97% (62 of 64). A 100% fertility was obtained in the fourth-generation rats from the control and both experimental groups. The body weights of the young were slightly higher in the experimental groups than in the control group; this would indicate that no interference with the lactation process had been occasioned by SC. The average weights of 3-day-old rats of all 4 generations were 6.9 g. for the control group, 7.0 g. for those receiving 1.9% SC, and 7.6 g. for those receiving 9.5% of SC in the diet. After 21 days, the comparable averages were 34.1, 35.4, and 37.0 g., respectively. Mortality from the 3rd to the 21st day was 15 for the control group, 5 for those which received 1.9%, and 2 for those which were fed 9.5% of SC.

In the 10-week growth tests, the averages of the mean weights of the 4 generations on the several dietary regimens showed little difference. The values for the male rats gave a grand average of 262.8, 261.1, and 255.9 g. for Diets 171, 187, and 188, respectively. In the case of the females, the corresponding grand averages were 172.7, 174.8, and 172.4 g., respectively. The mean weights of the rats after 10 weeks on both experimental diets containing SC were approximately identical, after 4 generations, with those of the first-generation animals on the same dietary regimen.

Growth Tests on Rats with Diets Containing Heated Fats Without or With Citric Acid Esters

Since it is known that some fats such as sardine oil become less completely digestible when subjected to a high temperature *(15)*, it was considered desirable to make tests on margarine fat heated without or with IC plus Vehicle or SC to determine whether a change in nutritional value may occur. In order to establish whether such a decrease occurs, growth tests were made on weanling rats using fats so treated as well as potato chips prepared by deep-fat frying in such fats. If the growth responses of the heated fats containing either of the citric acid esters tested were found to be inferior to that of the control heated fat, such an effect might be ascribed either to decomposition products of the citric acid esters or to a decomposition of the fat caused by heating in the presence of the citric acid esters. Data on these tests are summarized in Table 11.

No adverse effects on growth were noted in either male or female rats over 10-week periods when margarine fats heated at 205°C. continuously for 8 hours and used for deep-fat frying were incorporated in the diets, as compared with control tests in which unheated margarine fat was employed in similar dietary regimens. Moreover, when IC plus Vehicle or SC was added to the test oils prior to the prolonged heating procedure, and these oils were then subjected to deep-fat frying for the 8-hour period, no toxicity developed. This fact was demonstrated by identical growth curves of rats receiving diets containing the fat heated with additives as compared with those of rats receiving diets containing the heated fat without additives as well as the unheated fat without additives.

In addition, no differences in the growth of weanling rats were observed when they were fed diets containing potato chips fried in margarine fats with additives over that noted when the potato chips had been prepared in margarine fat without these citric acid esters. These results indicate

		[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	60	3
		3	19	17	27.7	4	7.7	7.2	33.6(35)	65	5
		4	16	16	26.6	5	7.1	6.9	34.5(63)	72	7
187	1.9	1	10	10	26.8	2	6.3	6.7	34.6(21)	41	0
		2	15	15	26.2	3	6.8	7.4	33.5(42)	53	0
		3	15	14	24.4	4	8.1	7.0	36.9(56)	83	3
		4	14	14	31.0	5	8.4	6.8	36.7(56)	71	2
188	9.5	1	11	11	30.2	2	7.6	7.0	34.5(43)	61	2
		2	23	23	26.2	3	8.5	7.2	35.5(105)	117	0
		3	10	14	26.5	4	8.5	8.5	41.0(56)	67	0
		4	14	14	27.4	5	9.4	7.6	37.1(84)	94	0

ee Period elapsing after the male and female rats were put in the same cages. dd Average weights of 7-litter rats only. Figures in parentheses give average number of rats. ee Experiment terminated before all rats were weaned.

TABLE 11

The Increase in Weight of Weanling Rats over 10-Week Periods Which Received Diets Containing Unheated Margarine Fat (Control), Margarine Fat Heated at 205°C. for 8 Hours Without or With IC plus Vehicle or SC, and Potatoes Fried in Such Fats

Diet No.	Category	Test material in diet[‡‡]	Number of rats	Body weight		Gain in weight	Total food eaten	Total test substance eaten	Efficiency of diet[§§]
				Start	End				
	Experiments on male rats								
		%		g.	g.	g.	g.	g.	
70	Unheated fat (Control)	0	10	35.4	258.2	222.8	819	0	6.68
73	Heated fat	0	10	36.2	265.5	229.3	758	0	7.08
89	Heated fat plus IC plus Vehicle	0.06	10	37.3	262.3	225.0	748	0.45	7.04
74	Potato chips from 73	0	10	39.9	260.4	221.4	758	0	7.43
90	Potato chips from 89	0.044	9	36.3	263.0	226.4	789	0.35	7.04
189	Heated fat plus SC	0.86	10	38.0	267.5	229.5	776	6.67	6.93
190	Potato chips from 189	0.68	11	39.0	265.1	226.1	793	6.40	6.68
	Experiments on female rats								
70	Unheated fat (Control)	0	10	39.7	180.2	140.5	701	0	4.93
73	Heated fat	0	10	38.1	187.0	148.9	670	0	5.20
89	Heated fat plus IC plus Vehicle	0.06	9	38.0	193.7	155.7	680	0.41	5.37
74	Potato chips from 73	0	9	37.2	182.1	144.9	677	0	5.42
90	Potato chips from 89	0.044	9	39.2	185.7	146.5	689	0.31	5.22
189	Heated fat plus SC	0.86	9	38.9	180.8	141.9	649	5.58	5.12
190	Potato chips from 189	0.68	8	39.4	181.3	141.9	699	5.65	5.00

‡‡ Based on content of citrate esters present in fat at start of heating. §§ (G. gain in weight/calories consumed) × 100.

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The authors wish to
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73	Heated fat	0	10	39.1	189.2	140.5	701	0	[illegible]
89	Heated fat plus IC plus Vehicle	0.06	10	38.1	187.0	148.9	670	0	4.93
74	Potato chips from 73	0	[illegible]	38.0	193.7	155.7	680	0.41	5.20
90	Potato chips from 89	0.044	9	37.2	182.1	144.9	677	0	5.37
189	Heated fat plus SC	0.86	9	39.2	185.7	146.5	689	0.31	5.42
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				39.4	181.3	141.9	699	5.65	5.12
									5.00

** Based on content of citrate esters present in fat at start of heating. ** (G. gain in weight/calories consumed) × 100.

that the heating of IC plus Vehicle or SC in margarine oil for 8 hours at 205°C. does not produce toxic decomposition products nor does it cause the oil to be adversely affected.

Acknowledgment

The authors wish to acknowledge the help of Dr. Wm. Baber, Mr. George Kryder, Mr. Lawrence Owens, and Mrs. Evelyn Savage during the course of the experiments.

SUMMARY AND CONCLUSIONS

The results of the present toxicological study of 2 new chemicals proposed for use as food additives may serve as an illustration of what constitutes adequate testing of new food chemicals. Mixtures of mono-, di-, and tri-alkyl esters of citric acid were evaluated for the safety of their use in stabilizing the flavor of oils and fats and foods containing such oils and fats.

Consideration was first given to an independent analysis on the part of the testing laboratory of the mixed esters of citric acid to check the claims made by the manufacturer that the products were actually what they were reputed to be. Because of the variations in composition of mixed esters, many different batches were employed in the current investigation; analyses were made of representative batches.

Consideration was then given to the possible hydrolytic products of the esters of citric acid, and evidence was cited from the literature showing that such hydrolytic products in themselves exhibit no toxicity. It was emphasized that the intact esters themselves must be subjected to exhaustive toxicological studies using 3 species, not more than 2 being rodents. LD_{50} values carefully determined on one species and checked with another, acute experiments on 3 species, longevity tests, multigeneration studies, and toxicological studies of the possible degradation products of the citric acid esters were all conducted. In addition, histopathological examinations of tissues obtained from sacrificed animals were made throughout the course of the investigation. When the major portion of the studies was completed, the data were presented to the Division of Pharmacology of the U. S. Food and Drug Administration for their criticism, and their suggestions were followed in carrying the toxicological investigation to completion.

More specifically, studies have been undertaken to determine whether 2 new flavor-stabilizing agents for fat products, namely isopropyl citrate esters, predominantly mono-isopropyl citrate (IC), the same plus mono- and diglycerides vehicle (IC plus Vehicle), and stearyl citrate esters, predominantly distearyl citrate (SC), possess any toxicity when measured by various appropriate indices.

The LD_{50} values for the 2 compounds studied were relatively high. That for IC plus Vehicle alone or dissolved in oil was approximately 19.0 g./kg. (7.2 g./kg. as IC), while the figure for SC exceeded 5.4 g./kg. in rats. No sex differences were noted.

There is no evidence from acute experiments on rats (6 weeks), rabbits (6 weeks), or dogs (11-12 weeks) of any toxicity as measured by growth, mortality, or pathological manifestations.

Longevity (chronic) tests were carried out on rats, for 2-year periods from weaning, on IC plus Vehicle at several levels (0.28, 0.56, and 2.80% of the diet) and on SC at 3 levels (0.5, 2.0, and 10.0% of the diet). No deleterious effects as compared with that of the controls were noted in any groups in so far as could be determined by growth rates, mortality, and histopathological examinations of tissues.

Another method for evaluation of possible chronic toxicity, namely multigeneration studies, likewise indicated that IC plus Vehicle, at a level of 2.8% of the diet, and SC, at levels of 1.9 and 9.5% of the diet, are completely innocuous. These conclusions are based upon fertility over 5 and 4 generations, lactation performance as judged by the 3-day and 21-day weights of the offspring, as well as by their survival for the nursing period, and by the rate of growth of rats of the several generations over a 10-week period following weaning.

Margarine fat containing 0.5% IC plus Vehicle or 7.5% of SC did not develop toxicity (as determined by rat feeding tests over 12 weeks) when subjected to deep-fat frying at 205°C. over an 8-hour period. Potato chips, fried in such fats, and which retained approximately 40% of the fat, were also entirely satisfactory for growth.

Histopathological examination of the tissues of the rats after 2 years, of rabbits after 6 weeks, and of dogs after 11 to 12 weeks on the test diets did not reveal any evidence of specific damage. In the case of rats and rabbits, the following tissues were subjected to histopathological examination: liver, kidney, gastrointestinal tract, spleen, heart, lung, pancreas, gonads, brain, and adrenals. Only the liver and kidneys were examined in the dog tests.

All tests for toxicity indicate that IC when given as IC plus Vehicle and SC are harmless at levels as high as 1.1 and 10% of the diet, respectively. The maximum level at which IC has been tested and proved to be harmless is more than 500 times that which would be ingested in margarine containing 0.02% of IC where this fat makes up 15% of the diet by weight. Similarly, the highest level at which SC has been tested and which has proved harmless is about 500 times greater than would be consumed when margarine containing 0.15% of SC makes up 15% of the diet by weight.

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