A VASOACTIVE POLYPEPTIDE EXTRACTED FROM CALLORHINUS MUSCLES, AND ITS CLINICAL APPLICATION

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We name it callopeptide, which is extracted from muscles and viscera of fur seal (*callorhinus ursinus*), because it is a polypeptide, according to an agreement now generally accepted^{8, 12} that peptide-like compounds of 3-90 amino acids are called polypeptide, of which the upper limit corresponds to a molecular weight of approximately 10,000 and the ability to pass through cerophane membranes,

Table 1
Classification of Functional Polypeptides

Classification	Examples	Amino acids	Function
1. Tissue hormones	1) Angiotensins	8	hypertensive
	2) Bradykinins	9	hypotensive
	3) Substance P	unknown	"
	4) Gastrin	17	secretory
2. Glandular hormones	1) Pituitary ACTH	39	secretory
	" Oxytocin	9	"
	2) Pancreas Insulin	51	glycolytic
	" Glucagon	31	"
3. Neurohormones	1) Corticotropin releasing factor	12	secretory
	2) Luteinizing hormone releasing factor	unknown	"
4. Antibiotics	1) Actinomycins	12 with a Rest	sterilizing
	2) Gramicidins	10 cyclic	"

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separates polypeptides from proteins.

The structure elucidation and pharmacological evaluation of biologically active polypeptides have greatly advanced in the course of the recent 10 years^{9, 11} as shown in Table 1 which is edited from the standpoint of physiology. Callopeptide belongs to a category of the tissue hormones in the table, according to its biological actions, especially among which to the hypotensive polypeptide group.

The hypotensive peptides hitherto discovered are summarized in Table 2. Their origins are various, for example, bradykinin is formed during active secretion in salivary glands, sweat glands, and exocrine portion of the pancreas, and it increases the blood flow in these tissues. Eledoisin is presumed to be the same. Other kinins are found in wasp and other insects venoms, they are not normal occurring but probably can be released in vivo. Substance U and Z must be also metabolite in vivo owing to the fact that they are found in normal urine. These hitherto-known active polypeptides are all metabolite in vivo, but callopeptide is not a metabolite, it is an artificially extracted polypeptide by a certain procedure from the skeletal and visceral muscles of a special marine animal.¹⁰

Table 2
Hypotensive Polypeptides and Their Origins

Name	Amino acids	Origin	Extraction	Authors
1. Bradykinin	8	human plasma	trypsin ethanol extraction	1947 Rocha e Silva (8)
2. Substance P	unknown	Equine intestine and brain	alcoholic extraction	1931 von Euler and Gaddum (5)
3. Eledoisin	11	Salivary glands of Cephalopoda	methanol extraction	1954 Erspamer (4)
4. Wasp-kinin	unknown	Wasp venom	diethyl ether extraction	1954 Jacques and Schachter (7)
5. Hornet-kinin	"	Hornet venom	"	1960 Bhoola Calle and Schachter (2)
6. Substance U	"	Urine	alcoholic extraction	1952 Belaldo (1)
7. Substance Z	"	"	"	1960 Werle and Erdös (15)
8. Colostrokinin	"	Bovine colostrum	hydrochloric acid, ethanol extraction	1960 Werle (14)
9. Pysalaemin	11	Amphibian skin	methanol extraction	1962 Erspamer, Bertaccini and Cei (3)
10. Callopeptide	unknown	Callorhinus muscles	protease, 60-80% alcoholic extraction	1963-1966 Hayashi <i>et al</i>

In order to determine whether this new polypeptide which has biological activity is present in other mammals, experiments were conducted on extracts prepared by the same method as used in preparing the callopeptide, using as the material animals, dog, cat, cow, hog and whale, common seal, dolphine, but the presence of an active ingredient could not be found. This result should be sufficient to assume that the callopeptide is a substance which is singularly possessed by the aquatic animals of the family otariidae. In other words, the existence of the substance in fur seal is quite akin to natural occurrence.

1. Extraction Method

The callopeptide is obtained by a method consisting of the steps (a) + (b), the step (a) being that in which at least one of either the skeletal muscles or viscera of a fur seal, an animal of the family otariidae, is decomposed and the step (b) being that in which the resulting decomposition liquid is extracted with a water-containing organic solvent, the water-containing organic solvent-soluble portion being collected.

Table 3
*Extraction Method**

1. Schredded, elimination of oils and fats mechanically and by hydrophobic solvent.
2. Protease, adding toluene to prevent microbes—4 hours.
3. Hydrochloric acid is added as to 10.8%—70°C 1 hour.
4. Neutralized, precipitates are removed by centrifuging, and barium hydroxide is added as to 4N.—65°C 1 hour.
5. Neutralized by sulfuric acid and precipitates are removed.
6. Boiled to inactivate the enzyme, and odorous substances are removed by steam distillation.
7. Concentrated under reduced pressure at a temperature as low as possible and ethanol is added until 60–80% by weight in 80–90°C.
8. Filtration and collection of ethanol-soluble portion.
9. Vacuum and spray drying,—an amorphous powder. (The crude product.)

* The industrial use of the method is covered by patents both in U.S.A., U.K. and other countries.

As shown in Table 3, in carrying out this decomposing step (a), first the material should preferably be shredded and eliminated oils and fats into a slurry state having fluidity by adding water in a suitable amount. Second when the decomposing is to be carried out by means of the enzymatic decomposition, a protease is utilized for several hours.

When the optimum pH of said protease is close to that of the material flesh,

it is used without adjustment, but if it is on the acid side, the pH of the material slurry is adjusted by adding a physiologically harmless weak acid.

The aforesaid decomposing step (a) can also be carried out by subjecting the material slurry to hydrolysis. Usually hydrochloric acid is added to a slurry of the material flesh in an amount such that the total hydrochloric acid concentration become 10–20%, the hydrolysis then being carried out for from several ten minutes to several hours, with stirring while maintaining a temperature of 30–80°C. After the decomposition, the liquid is neutralized by adding caustic soda, following which the precipitate is removed by centrifuging.

Then in carrying out the extraction step (b) the following procedure is followed. The decomposition liquid is boiled to inactivate the enzyme followed by filtering off the solid matter and, the remaining organic solvent and odorous substances are then removed from the decomposition liquid usually by means of steam distillation. The solution is then concentrated under reduced pressure at a temperature as low as possible, after which ethanol is added to the concentrated liquid, if necessary, with the addition of water, until the concentration of said ethanol in the liquid after addition of the solvent becomes 60–80% by weight. The liquid is then heated under reflux preferably at 80–90°C. to effect the full dissolution of the soluble portion in said organic solvent, following which filtration is carried out to collect the ethanol-soluble portion.

The treatment of collecting the water-containing extraction solvent-soluble portion may be repeated. When the resulting extraction solvent solution is dried utilizing evaporation, vacuum and spray drying or other known drying techniques, an amorphous powder containing the callopeptide in high concentration is ob-

Table 4
Physical Properties

1. Soluble in
(1) water
(2) water-containing ethanol (not more than 80%)
(3) methanol, butanol
(4) acetone (water-containing at least 20%)
Insoluble in
(1) alcohols of above 80%
(2) acetone of above 80%
2. Dialysable through cerophane membrane. (The inside liquid loses physiological actions.)
3. Molecular weight
(1) positive Biuret reaction indicates that it is greater than tripeptide.
(2) Swedberg unit is not more than 1.0, estimating as being S_{20} , hence M.W. is not greater than 10,000.

Table 5
Chemical and Biological Properties

1. Amount of free amino acids was negligible.
2. The number of unknown peaks of Abderhalden-Schmidt reaction was 10-12.
3. Hydrolysis; peaks disappear and amino acids, roughly estimated, appear as following; glycine, alanine, valine, leucine, isoleucine, proline, threonine, lysine, aspartic acid, and glutamic acid.
* LD ₅₀ of callopeptide via peritoneal injection
CP = 14.4 g/kg PP = 7.7 g/kg (mouse and rat)

tained, which we named the crude product (SP). The crude product has the following physical and chemical properties as shown in Table 4 and 5.

- (1) Solubility: Soluble at room temperature in water and water-containing ethanol (not more than 80% ethanol). Soluble at room temperature in methanol, butanol and acetone (containing in all cases at least 20% of water). Insoluble in alcohol and acetone of above 80% concentration.
- (2) Biuret reaction: The biuret reaction to be hereinafter defined is positive. The maximum absorption of the color resulting from the biuret reaction is at 550 m μ . In this connection that in the case of native peptide is 545 m μ .
- (3) Estimation of molecular weight: A cellophane tube is filled with 100 ml of a water-contained alcohol solution containing about 10 mg of peptide per 1 ml of the solution, following which dialysis is carried out with water for 24 hours at 20°C. From the fact that the inside liquid of the dialysis, when tested for its physiological activity, had lost its activity, it is not conceivable that said peptide is a polypeptide having 10 or more amino acids linked thereto. That the biuret reaction is positive indicates that it is tripeptide or greater. An aqueous solution containing about 8 mg of peptide per 1 ml of the solution is ultracentrifuged at 60,000 rpm for one hour. When the resulting schlieren pattern is observed, the sedimentation constant in terms of Swedberg units is estimated as being S₂₀ (sedimentation constant at 20°C) of not more than 1.0, and hence it is estimated that the molecular weight is not greater than 10,000.
- (4) Tyrosine residue: The optical density at 220-230 m μ at which is seen the absorption of the tyrosine residue of a 0.005 $\pm$ 0.001% by weight aqueous solution of the substance is substantially zero.
- (5) Amino acids: When the substance is tested with the amino acid automatic analyzer, the amount of free amino acids is very slight, and number of unknown peaks which develop color by reacting with ninhydrine (Aberder-

halden-Schmidt reaction) are observed to be 10–12 or even more. When the callopeptide is subjected to hydrolysis and then again tested with the amino acid automatic analyzer, these peaks disappear and practically all classes of amino acids appear.

2. The Purification

In order to further purification, the crude product in ethyl alcohol solution is concentrated under reduced pressure to eliminate the alcohol as well as to concentrate the original solution to a volume 1/100–1/50 of that of the original volume, following which the solution is passed through a column of cross-linking dextran for example, "Sephadex" G-10, G-25 and G-50 (products of Sweden-Pharmacia Company) those with the smaller G-number being the finer and the biuret reaction positive portion is collected.

In carrying out this treatment, the upper end of a column packed with "Sephadex" G-25 (fine) is filled with the previously described concentrated liquid, after which it is flowed down through the column using distilled water. An outflow rate of about 25–75 ml per hour is suitable. When, for instance, 5 ml portions of the liquid are fractionated. A biuret reagent is added to the liquid and the optical density of the color developed is measured at a wavelength of 550 $m\mu$. At the same time the optical density shown by the tyrosine residue at a wavelength of 270–280 $m\mu$ is also measured.

When this is illustrated with reference to Fig. 1, the optical density at the wavelength of 550 $m\mu$ is indicated on the vertical axis at the left, while the optical density at the wavelength of 270 $m\mu$ is indicated on the vertical axis at the right. On the other hand, the horizontal axis shows the volume of the liquid

Fig. 1 Fractioned portions and their optical densities at 550 $m\mu$ and 270 $m\mu$ wavelength.

which has flowed out, the numerals being the test tube number of fraction collector, each of which has a capacity of 5 ml. The curve shown by means of the solid line is the optical density curve of the instance when the specimen was measured at the wavelength of $550\text{ m}\mu$, while that shown by means of the dotted line is the optical density of the instance when the specimen was measured at the wavelength of $270\text{ m}\mu$. The ranges in which test tubes Nos. 1—12, 13—28, 29—35, 36—44 and 45—50 fall are considered to be respectively the first, second, third, fourth and fifth fractional portions.

The optical density of both curves appear intensely in the second and third fractional portions, the indication of biuret reaction positive being especially pronounced. When the physiological activity of the several fractional portions are investigated, they are as follows: A strong capillary vasodilator activity was noted in the case of the second and third fractional portion (purified product PP). It could not be confirmed in case of the portions other than the second and third fractional portions. From the fact the components of this second fractional portion especially shows an intense biuret reaction, it is presumed that it contains polypeptides of tripeptide and above. Further, when components of this portion are examined with the amino acid automatic analyzer, the free amino acids are observed in very minute amounts, but there are seen 10—12, or more, unknown peaks which develop color by reacting with ninhydrine. Next, when this portion is subjected to hydrolysis and again examined with the amino acid automatic analyzer, these unknown peaks disappear, and practically about 10 amino acids appear, as shown in Table 5. From these results, it is presumed that there is present together in the second fractional portion a number of classes of polypeptides, but these 10 amino acids combined one will be most concentrated.

As one of the other purification means, there is a method wherein an ion exchange resin is used. In this case, the aqueous solution from which has been evaporated under reduced pressure the solvent of the water-containing extraction solvent solution, or an aqueous solution of the crude product which has once been made into a powder is eluted with an acid buffer using a column of an ion exchange resin such, for example, as "Dowex" 50 or "Amberlite" 50, following which the effluent is collected and concentrated to a given concentration.

3. Actions of Callopeptide on the Brain of Mammals

To study the biological action of callopeptide, we first determined the lethal dose for mice or rats, by the peritoneal injection. The lethal dose, LD 50, was 14.4 g/kg in the crude product and 7.9 g/kg in the purified one, as shown in Table 5. In later experiments, we used 1-2 mg of 1.0% solution for cat and rabbit, 1-2 mg of 2.0% solution for dogs.

Table 6

EEG and Evoked Potential

1. EEG in dogs—no change for 2 hours when 2 ml/kg of 1.0% of the crude callopeptide (C.P.) solution was given through peritoneal injection.
2. Evoked potential of cats—10–17% enhancement, 30–135 min after the peritoneal injection of 2 ml/kg of 1.0% crude callopeptide solution.
3. Dilatation of surface blood vessels of exposed cortex of dogs after the peritoneal injection of 2 ml/kg of 1.0% callopeptide. When the preparation was for 4–5 hours surgically manipulated one, the dilatation accompanied a diffuse bleeding to a slight oozing extent.

Fig. 2 The evoked potentials of the cortex of a cat, before and after the callopeptide injection.

Fig. 3 The membrane potentials of the ipsilateral somatic sensory cortex of the cat, before and after the callopeptide injection.

The first experiment was to try the measuring EEG of dogs when callopeptide of 2 ml/kg of 1.0% of the crude product solution was given by peritoneal injection. As Table 6, the cortical EEG has shown no change for 2 hours after the injection.

The second experiment was to measure the evoked potential of cortical cells in dogs. A microelectrode was applied on the somatic sensory area of dogs and its evoked potential was measured by the electrical stimulation to the excised ischiadicus nerve. Fig. 2 showed the evoked potentials when callopeptide was injected intravenously. The potential measured at the contralateral somatic sensory cortex was increased 15—17% of height as shown in Fig. 2. On the contrary the ipsilateral somatic sensory area has not shown the evoked potential, of course, before the injection as in Fig. 3, nor after it.

Not only the evoked potential was augmented the surface blood vessels of the cerebral cortex were distinctly dilated by naked eye observation, and most significant phenomenon was the diffuse bleeding from the surface vessels of cerebral cortex in the preparation which was undergone through a long surgical manipulation.

Thus we have obtained three notices.

1. An augmentation of the evoked potential with a latent period of 20—40 min after intravenous injection of callopeptide, and it continued for hours.
2. Dilatation of peripheral vessels.
3. Permeability of general capillary walls increased.

Table 7
Action of Callopeptide upon Cortical Cells

1. Enhancement of evoked potential (see Fig. 2 and 3)			
2. Dilatation of peripheral blood vessels.			
3. Permeability of capillary walls increase. For example a diffuse bleeding after a lapse of long surgical manipulation.			
Does the enhancement of evoked potential of the cortical cells come from			
1) Stimulatory action of callopeptide, or			
2) The dilatation of cortical blood vessels?			
Dog	Callopeptide into csf (each 5 min)		Motor action (Seizure)
male 10.0 kg	10% solution	0.5 ml	(—)
	"	0.5 "	(—)
	"	0.5 "	(—)
	"	1.0 "	(—)
	"	1.0 "	(—)
	"	1.0 "	(—)
	"	1.0 "	(—) no further change.

Does the augmentation of the evoked potential come from (1) stimulatory action of callopeptide on cortical cells or (2) from the dilatation of cortical blood vessels? To solve the question, we made an experiment to introduce the substance into cerebrospinal fluid of dog. If the substance has a stimulatory action, when we introduce the substance excessively into c.s.f. of the animal it must excite the motor cortex and produce seizure. Table 7 showed the experiment that the motor phenomenon could not be obtained when 5.0 ml of 10% of callopeptide solution was introduced. Moreover, when we applied the solution on the exposed cortical surface, it did not excite the cells. Thus the augmentation of the evoked potential of cortex, presumed to be owed to the dilatation of cortical vessels, not to the stimulatory action of the substance.

As histamine is vasodilatory substance, we compared the action of the dilatation on cats cortical vessels of callopeptide and histamine. Table 8 showed the results. Callopeptide was effective when it was given as to the concentration of 0.000143% in cats body, the congestion of cortical surface was obtained, and vascular permeability increased, but in histamine when its concentration was attained to 0.00089—0.0089% the same dilatation was obtained, and it was over than the lethal dose for the cat.

Table 8
Callopeptide and Histamine upon Cortial Vascular System

Items	Callopeptide	Histamine
Animal	Cat (2.5 kg)	Cat (2.5 kg)
Dose of peritoneal injection	1 ml of 0.358% CP	1 ml of 0.2-2.0 M
Calculated concentration	0.000143%	0.00089%—0.00889%
Congestion	+	+
Diffuse bleeding (after a long surgical manipulation on the exposed corotex)	+	—
End effect	alive	dead

4. Vasodilatory and Hypotensive Action of Callopeptide

Vasodilatory action of callopeptide was experimented i) to examine the mesenterial blood vessels by abdominal window method in rabbits, and ii) to observe the vascular beds of rabbit's retina by taking colored pictures before and after the intravenous injection of 2 ml/kg of 1.0% callopeptide solution.

An example of retinal funduscopy is shown in Fig. 4, A, B, C and D. Fig. 4A was a control picture before the injection and B the one hour later when 0.9 ml/kg

of 2.0% callopeptide solution was intravenously injected. Comparing the colors of both fundus pictures, the vasodilating action of callopeptide can be recognized by naked eye.

Fig. 4 Pictures of retinal funduscopy of a rabbit. Rabbit 2.8 kg. A: before the injection, B: 60 min, C: 120 min, and D: 180 min after the injection of 0.9 ml of 2.0% callopeptide solution.

From our studies, callopeptide has a strong vasodilatory action in cortical surface, mesentery and retinal fundus. Generally speaking, the peripheral vessels of the whole body should be dilated by callopeptide. Necessarily it must result the blood pressure lowering. Then, hypotensive action of callopeptide was examined by the measuring of blood pressure of A. femoralis by inserting a cannula into the left femoral artery of a rabbit. The normal pressure was 150–160 mmHg in 2.5–3.5 kg weighed rabbits, and when 2 ml/kg of 1.0% callopeptide solution was given intravenously, the pressure was lowered about 20 mmHg with a latent period of 30–40 min as shown in Table 9. There was an upper limit of the effect of callopeptide, for example, when the callopeptide was given 5–7 ml/kg of the above solution, the pressure could not be lowered more than 25 mmHg.

Table 9
Vasodilatory and Hypotensive Action of Callopeptide

1. The vascular beds of rabbits retina, was taken by colored picture before and after the intravenous injection of 0.9 ml/kg of 2.0% callopeptide solution. (Fig. 4 B and C)
2. Blood pressure of carotid artery of rabbits.
normal—150–160 mmHg, 20 mmHg lowering after the peritoneal injection of 1–2 ml/kg of 1.0% solution of callopeptide (C.P)

Any more lowering of the blood pressure was not seen up to the lethal dose.

The evidence that the callopeptide belongs to the group of hypotensive tissue hormones in Table 1, would be made quite definite, by the comparison of physiological actions with that of bradykinin, as shown in Table 10.

Table 10
Comparison of Physiological Actions between Bradykinin and Callopeptide

Action	Bradykinin	Callopeptide	
Hypotesnsive action	strong	medium	
Contraction of visceral smooth muscle	+	slight	
Dilatation of vascular smooth muscle	+ with some controversies	+	(Cat, Rabbit Rat)
Permeability of capillary	increase	increase	
Pain producing action	severe pain	severe pain	(Human)

Hypotensive action of both substances is different, it is strong in bradykinin and weak in callopeptide. Action to produce contraction for visceral smooth muscle is also strong in bradykinin, but it is very weak in callopeptide. Action of dilatation of vascular smooth muscle is strong but instant in bradykinin, on the contrary it is as well strong in callopeptide and it continued rather constant and for several hours. Still more the dilatatory action of vascular muscle due to bradykinin is much controversial. Some authors believe that bradykinin makes vein contraction, and its necessarily sequence the calillaries will be filled by blood, accordingly small arteries will be secondarily dilated.^{6, 13} But in callopeptide it seems to make arterioles, as well as venules dilate, consequently blood flow increases, first locally in the applied area and second in the peripheral vessels of the whole body.

The last problem was a disclosure that bradykinin could not be absorbed through the rabbit's skin, but callopeptide will be absorbed. Table 11 shows that the threshold dose of callopeptide to dilate the blood vessels of mesentery examined by the abdominal window method was 0.06—0.08 mg/kg for a 2.5 kg rabbit. The dose was calculated as 0.006—0.008mg% for the rabbit's body. When an area of 2×20 cm was shaved in rabbit's both breast and coated by callopeptide containing cream of each 1 ml. The cream contained 0.07—0.11% callopeptide. In both sides, totally 1.4—2.2 mg was used. Sixty to one hundred twenty minutes after paint, the blood vessels were dilated, in other words, through the skin the threshold amount to make dilatation was absorbed in the lapse of the

Table 11
Vasodilatory Action of Callopeptide through the Abdominal Window Method

Rabbit 2.5 kg	Intravenously application	Application on skin 2×20 cm diameter areas in both breast
Threshold dose of vasodilation	0.06-0.08 mg (pp)/kg	1.0 ml of 0.07-0.11% (PP) in dressing cream in both breast
Latent period	20 min	60—120 min
Calculated concentration	0.006-0.008 mg%	—
Absorption rate	—	4.28—5.71%/60 min

time. It must be calculated as 0.006—0.008 mg%. In the skin area, there were 1.4—2.2 mg callopeptide, of which 4.28—5.7% amount was absorbed.

Thus the absorption rate of callopeptide through hair shaved skin area of 4×20 cm of a rabbit has been obtained, and it is shown in next section, by another example in human by clinical application.

5. A Few Trials of Clinical Application

We confirmed first that callopeptide had no action to promote nor inhibit the cancer cell proliferation. The experiment was carried out with mice (dd N strain) in which cancer cells S-180 was transplanted into peritoneal cavity and the amount of 1.0 ml of 10% callopeptide per kg was daily injected for 5 days in the experimental mice group. Along for 2 weeks in the control group as well as in the experimental group, the weight increase were quite same and all died in the same day. This was the preliminary base for the clinical application.

CASE 1. A 65 years old Japanese male was hospitalized on April 4, 1966, for complaint of pain in his legs for several months. A physician in charge, diagnosed neuralgia ischiadica with diabetes mellitus and cataracts. The disease

are very popular in Japan and this was a typical one.

Immediately after hospitalization, Vitamin B₁ and aspirin and several times injection of narcotics a day were given to prevent the acute pain. And after 5 months of without success the doctor tried to use an ointment made by himself, which contained 1.0% of callopeptide (PP) to let the patient to paint at his both loam to legs expanding the medicament of about 5 ml, two times a day. To his surprise the pain of legs disappeared within 2 days and continued for next 6 hours. Third day, by the earnest beg of the patient, he gave the same treatment, and noticed that the warmfeeling taking the place of pain, in the lapse of 20—30 min, and it continued for 6—7 hours.

The treatment was fixed hereafter to give the ointment every 6 hours, and the patient was treated in such a way for 4 weeks, and it was without pain, and without sleeplessness. The doctor stopped the treatment and without any suggestion he painted the same looking ointment without callopeptide. The patient complained to doctor for a weak pain next day. After 3 days the patient felt severe pain in his legs and the doctor again treated him with callopeptide for 2 weeks. Two to three repetitions of such a procedure, the patients said that he had pain in these intermittent days, but gradually the pain has become weak and the different endurable one. The treatment is now continued as in Table 12.

Table 12
Clinical Cases treated by Callopeptide

Cases sex, age	Symptoms	Diagnosis	Treatment	Result
Case 1 male 56	pain attacks in legs	neuralgia icshia- dica with diabetes mellitus	3 days pain disapperaed	8 weeks, weak pain without callopeptide
Case 2 female 47	severe pain in left thigh and left leg	neuralgia icshia- dica with spondylo- lysis deformans	2 days pain disappeared	8 weeks, pain is remained in left sole
Case 3 male 34	severe pain in right shoulder and right arm	periarthrits humeroscapularis (Dupley's disease)	2 days pain disappeared	8 weeks, com- pletely recovered

CASE 2. 48 years old female, complained pain of left thigh and leg and was hospitalized at May 13th 1966. The physician in charge diagnosed spondylolysis deformans (osteoarthritis) with neuralgia ishiadica, on the base of Röntgen examination. Till October, all trials were done to inhibit pain without success, the doctor at last applied 1.0% callopeptide to paint the leg from the groin to the foot with 5 ml of the ointment. The patient felt very warm within

20 min. And the pain disappeared after 2 days. She was lame in the left leg since a year, but now she could walk without lame after two weeks callopeptide treatment. Amount of the ointment was reduced to half dose recently, but she had no pain for 6—7 hours after the paint.

The intermittent period between treatments was made longer, at first 2 days between 2 weeks, then 4 days to 6 days. The pain of these days became endurable, till the end of 6 days, some severness returned. The beggining of December 1966, the pain was quite disappeared, except at her foot sole of the left leg, localised in about 5 cm × 3 cm.

CASE 3. 34 years old male patient, the diagnosis was periarthritis humero-scapularis (Duplay's disease). The pain attacks precipitated several times a day, and he visited the hospital in the end of September 1966. One month trials of various treatment, the doctor prescribed callopeptide, to paste it through shoulder of right side to the arm with 4 ml a time, two times every day. At first the pain did not disappear till the third day (about 50 hours). But the pain has faded away gradually a step by step and the third day evening the patient found that he did not feel pain in his arm. All treatments hitherto tried were given up and the treatment continued 4 weeks, and stopped. The pain returned after 12 hours, but the patient felt a quite weak, and in a short time all pains disappeared. The treatment now discontinued for one month.

6. Discussions and Prospects

Summarization of the above study has been already discussed in section 4. We have left many problems to be further studied, which were enumerated in Table 13.

The purification must be repeated, first to confirm the N terminal at the same time C terminal of the callopeptide, but the ultimate structure is only to be

Table 13
Consequential Presumptions to be studied

-
1.	The further analysis of the purified product.
2.	The synthesis and identification.
3.	In what species of animal besides Fur Seal does it naturally occur?
4.	The further studies of its physiology.
	1) Relation between bleeding and vasodilatation due to callopeptide.
	2) Callopeptide and catecholamins.
	3) Callopeptide upon blood clotting.
	4) Callopeptide and plasmin or antiplasmin.
5.	The further studies of clinical applications.
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attained by identification through a contrivable synthesis.

In one hand the further studies from the standpoint of physiology must be carried out and solve several problems, for example, whether the bleeding after a long surgical manipulation can be seen also in other areas, and its relation to vasodilatation due to callopeptide. Any effect does have callopeptide upon the blood clotting, or on the thrombus dissolution by plasmin. Are there antivaso-active substance contra callopeptides? Concerning to its action of cathecolamin on dilatation due to the callopeptide must be elucidated.

Table 14
Episodic Experiences remained to be Studied

A. External applications for	
1)	Writer's cramp. Occupational Raynaud's syndromes
2)	Raynaud's disease—Gangrene
3)	Neuritis due to metabolites
4)	Rheumatoids
5)	Skin diseases
	(1) Acne
	(2) Freckles
	(3) Aged flecks of face
	(4) Grey hair turns black
	(5) Stop of baldness
	(6) Keloid
	(7) Eczema
6)	Skin culture—cosmetic application
B. Internal applications for	
1)	Fatigue
2)	Sleeplessness
3)	Renewal of vitality

The biological importance, is in the question, in what species of animal besides fur seals does the callopeptide naturally occur? Because we could not believe that the substance is contained in fur seals muscles only.

The further studies of clinical applications must be extended to several diseases as in Table 13, from the principle that the local vasodilative action can reinforce tissue activities, among them, to Raynaud's disease, including occupational Raynaud's syndrome and writer's cramp which deserve to try by the external use of the substance.

There have been episodic experiences in our surroundings as shown in Table 14, but these must be experimentally elucidated. First, by the external application of the substance on skin diseases, for example 1) acne 2) keloid and

3) some eczema. Second, also the external application is for skin culture. Exploitation of callopeptide containing dressing creams for cosmetic use already has been progressed in Japan since 2 years. If it might be absorbed through alimentary canal up to some grade without decomposition, as it is absorbed through skin (Table 11), we can apply it to cure general fatigue or sleeplessness.

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