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ON
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ABBREVIATIONS

ACh	Acetylcholine
ATP	Adenosine triphosphate
CNS	Central nervous system
H	Histamine
5-HT	5-Hydroxytriptamine (Serotonin)
i. p.	intraperitoneal(ly)
i. v.	intravenous(ly)
s. c.	subcutaneous(ly)
SP	Substance P

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CONTENTS

Preface
Prof. *V. Butozan*
Opening Adress to the Symposium
Prof. *E. Grin*
Chairman's Opening Remarks
Prof. *P. Stern*

A. Moderator: Prof. Marthe Vogt

I. BIOLOGICAL TESTS
J. H. Gaddum,
The Estimation of Substance P in Tissue Extracts . . . 7

II. PURIFICATION (UNITS)
S. Huković, R. Košak and P. Stern,
Parallel Central Effects of 5-Adenylic Acid and Substance P Preparations of Various Potencies 15

III. ACTIONS
K. Umrath,
The Relation of Substances P to Neurotransmitter substances 23
H. Caspers,
Some Actions of Substance P on the Cerebral Cortex and the Brain Stem Reticular Formation 29
B. Pernow,
Some Effects of Substance P in Man. I. Effects on the Circulation. II. The Effect on the Intestinal Motility . . 45
B. Pernow and U. S. v. Euler,
Effect of Intraventricular Administration of Substance P in the Unanaesthetized Cat (Film) 53

B. Moderator: Prof. J. H. Gaddum
F. Lembeck,
Lack of Interaction Between Substance P and Drugs on the Intestine 55
B. Pernow and S. Wallensten,
The Relationship Between Substance P and the Motility of the Small Intestine in Man 57

ANU BiH АНУ БиХ

D. B. Beleslin and B. Ž. Radmanović,
The Effect of Substance P on the Isolated Superior Cervical Ganglion of the Cat 61

V. Varagić, D. B. Beleslin and B. Ž. Radmanović,
Some Peripheral Neurotropic Effects of Substance P . . 65

Radmila Pržić,
The Influence of Substance P on Attack Against the »Writhing Syndrome« in the Mouse 71

R. Milin,
The Influence of Substance P on the Nervous System of the Earth Worm (Lumbricus terrestris) 75

IV. SPECIFIC ANTAGONISTS

P. Stern and S. Huković,
Specific Antagonists of Substance P 83

C. Moderator: Prof. K. Lissák

V. DIFFERENTIATION FROM OTHER SUBSTANCES

G. Zetler,
Two Hitherto Unknown Biologically Active Polypeptides in a Substance P Preparation Made from Cattle Brain . . 89

VI. DISTRIBUTION

F. Lembeck,
Aspects Concerning the Occurrence and Distribution of Substance P in the Brain 97

J. Franz, R. A. Boissonnas and E. Stürmer,
Pharmacological Properties of Substance P Isolated in an Apparently Pure State 99

U. S. v. Euler,
Distribution and Action of Substance P in Fish and Invertebrates 103

U. S. v. Euler and F. Lishajko,
Presence of Substance P in Subcellular Particles of peripheral Nerves 109

Danica Kocić-Mitrović,
Substance P in Pregnancy 113

D. Moderator: Prof. U. S. v. Euler

VII. COMPARISON WITH OTHER BRAIN POLYPEPTIDES

K. Lissák and E. Endrōczi,
The Inhibitory Substance of the Brain 117

ANU ВиХ АНУ БиХ

CONTENTS

A. Carraro, F. Clementi, F. Fraschini, L. Martini and E. Müller,
Neurohumoral Control of the Anterior Pituitary Gland . . 123

W. A. Krivoy,
A Comparison of the Actions of Substance P and Other Naturally Occurring Polypeptides on Spinal Cord . . . 131

VIII. INACTIVATION

P. Stern, Ivanka Gašparović and Julijana Kovač,
A Factor Inactivating Substance P Present in the Serum of Pregnant Women 139

ANU BiH АНУ БиХ

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BiH
АНУ
БиХ

PREDGOVOR

Naučno društvo Narodne Republike Bosne i Hercegovine, među ostalim svojim zadacima, u svoju delatnost ubraja i održavanje kako nacionalnih tako i internacionalnih simpozijuma o aktuelnim naučnim problemima.

Simpozijumi, ukoliko se solidno pripremaju, a naročito diskusije koje se razvijaju u vezi s referatima podnesenim na simpozijumu, znače, bez sumnje, značajni doprinos naučnom rešavanju postavljenog problema.

Polazeći s ovog stanovišta, Naučno društvo je smatralo da je korisno organizovati međunarodni simpozijum o supstanciji P, s obzirom na aktualnost ovog problema i dosadašnja eksperimentalna istraživanja o značaju i ulozi ovog interesantnog polipeptida i transmitora u psihijatriji, farmakologiji, okulistici, fiziologiji, histologiji, patofiziologiji, pedijatriji i internoj medicini. Ova supstancija kao prenosilac senzibilnih i inhibitornih neurona i medijator sprovođenja aferentnih nervnih impulsa pobudila je znatan naučni interes u medicinskim i u krugovima biohemičara u poslednje vreme, naročito u vezi s normalnim i patološkim funkcijama centralnog nervnog sistema.

Na Medicinskom fakultetu u Sarajevu, u Farmakološkom institutu, vrše se sistematska istraživanja o ovoj interesantnoj supstanciji već duže vremena. Rezultati ovih proučavanja su već publikovani i na taj način postali pristupačni širem krugu medicinskih istraživača. S druge strane, saradnici pomenutog Instituta s velikom pažnjom prate dostignuća istraživača u drugim naučnim institucijama, kako u Jugoslaviji tako i u inostranstvu. Isto tako su istraživači sa sarajevskog Instituta stupili u lični kontakt s drugim istraživačima u cilju izmene iskustava i dostignuća o ulozi supstancije P.

To je bio razlog što je Naučno društvo poverilo svome Odeljenju medicinskih nauka organizovanje međunarodnog simpozijuma u uverenju da će rad na njemu doprineti osvetljavanju značaja ove važne supstancije ne samo s naučnog gledišta već i sa strane njezinog praktičnog značaja u medicini.

Smatramo da je naše Odeljenje medicinskih nauka uspelo da zainteresuje eminentne istraživače ovog problema iz brojnih zemalja i da ih privoli da uzmu učešća na simpozijumu bilo sa značajnim referatom bilo u konstruktivnoj diskusiji. Učesnici iz osam zemalja osim Jugoslavije opravdali su svojim istupanjem nadu organizatora simpozijuma da

će svojim referatima i rezultatima svojih eksperimentalnih radova doprineti osvetljavanju problema supstancije P iz različitih aspekata.

Zahvaljujući upornom zalaganju organizatora simpozijuma, a naročito profesoru dr Sternu i njegovim saradnicima, Naučno društvo smetra da je uspelo u svojoj nameri, o čemu najbolje svedoče publikovani radovi i diskusija na simpozijumu u ovoj skromnoj svesci. Nadalje smatramo da će ovaj međunarodni simpozijum i rezultati postignuti na njemu korisno poslužiti uzajamnom upoznavanju i korisnoj razmeni rezultata rada naučnih radnika iz različitih zemalja, što, u krajnjoj liniji, po našem mišljenju, služi unapređivanju nauke uopšte.

Prof. dr Vaso Butozan

Predsjednik Naučnog društva
Bosne i Hercegovine

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PREFACE

The Scientific Society of the People's Republic Bosnia and Herzegovina counts to its activities, among others, the sponsoring of scientific meetings on topical problems at national as well as at international levels. Such Symposia, and particularly the discussion of the papers, doubtlessly represent significant contributions to the solving of scientific problems.

From this view-point the Society estimated that the organizing of an international Symposium on Substance P would presently be useful, in consideration of the timeliness of this topic, as well as of the host of experimental research of the rôle played by this very interesting polypeptidic transmitter substance which has been hitherto carried out in various fields, e. g. psychiatry, pharmacology, ophtalmology, physiology, histology, pathophysiology, pediatrics and internal medicine. As a sensory transmitter and a mediator in inhibitory processes this substance has recently encountered a considerable interest among physicians and biochemists, particularly in connection with normal and pathological functions of the CNS.

There are systematic investigations in the SP field going on for some time at the Institute of Pharmacology of the Medical Faculty of Sarajevo. Results of these investigations have already been published and made available to a larger number of investigators. On the other hand, workers in this Institute follow carefully the results reported by other authors in this country and abroad, and they have also exchanged experiences in personal contacts.

The Scientific Society has, therefore, entrusted its Department of Medical Sciences with the arrangement of a Symposium on SP in Sarajevo, deeming that such a meeting would afford further opportunity for the interchange of views, and that its proceedings would contribute to the elucidation of various rôles of this important agent. It was hoped that all this would take place not only at a purely theoretical basis, but would also be valuable with regard to practical utilization in medicine.

The efforts of the Department have apparently been successful in rousing the interest of eminent investigators from several countries for the present Symposium and obtain their participation in presenting papers and taking part in constructive discussion. Participants from eight countries, besides this country, have, through their contributions

fulfilled our hopes in clarifying various problems around SP by treating them from different aspects.

So thanks to the efforts of contributors, but also to the persevering endeavors of the Organizing Committee, particularly professor Stern and his collaborators, the Society esteems to have attained its purpose regarding this Symposium, which is best reflected in the papers and discussions contained in this booklet. I think that this Symposium and its results have also been useful to a better mutual acquaintance and exchange of experiences between scientists from several countries and this, in my opinion, ultimately serves to the advancement of Science in general.

Prof. Dr. Vaso Butozan
President, of the Scientific Society
of Bosnia and Herzegovina

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OPENING ADRESS TO THE SYMPOSIUM

Professor Ernest Grin, Vicepresident of the Scientific Society of the People's Republic of Bosnia and Herzegovina:

Distinguished Guests, Ladies and Gentlemen,

It is my great pleasure and honour to extend on behalf of the Scientific Society of People's Republic of Bosnia and Herzegovina first a sincere welcome to our distinguished guests who are representing authorities of our Republic:

Prof. Edhem Ć a m o, President of the Council of Science of the People's Republic of Bosnia and Herzegovina; Prof. Aleksandar T r u m i ć, Rector of the University of Sarajevo; Prof. Ivo H e r l i n g e r, Dean of the Medical Faculty of the University of Sarajevo; Prof. Bogdan Z i m o n j i ć, President of the Medical Association of Bosnia and Herzegovina.

It is further a great pleasure for me to express a warm welcome to all participants and particularly those from foreign countries — United States, United Kingdom, Austria, Italy, Hungary, Sweden, Switzerland and Germany — who represent the most outstanding scientists in the field of pharmacology of international significance.

Our Society considers it a great honour that this Symposium is being held in our City and we greately appreciate that the participants have accepted our invitation to take active part on this meeting.

We are also grateful to the authorities of our People's Republic and to all institutions for their generous aid which made it possible to arrange this international Symposium.

Medicine is not a subject confined to the borders of any single country and impulses which scientific research receives through international meetings are of the utmost importance in clarifying and analysing the results obtained which will often be highly valuable making it possible for the medical achievements of each country to be added to the common good of all humanity.

In view of the more and more complicated procedures of modern science and experimental research, a collaboration among the individual investigators and research institutions has become almost imperative, and to promote and stimulate this collaboration and the interchange of ideas and concepts by sponsoring, Symposia of a national and international character, such as that being held here today, is one of the most distinctive aims of our Scientific Society.

In facilitating this the question of a complete publication of the Proceedings of this Symposium has been also considered by the Society and it has been decided to do so as soon as all manuscripts of the Transactions will be in the hands of the Editor. The papers should be published in English, with summaries in Serbo-Croatian, but if you may wish otherwise, our Society will be glad to reconsider this question.

In the conviction that experiences which will be presented here will promote our clinical abilities and provide fresh impulses for further research work I wish a most successful course to the Symposium and we shall do our utmost to make your stay here as interesting, profitable and pleasant as possible.

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P. STERN

CHAIRMAN'S OPENING REMARKS

Thanks to the extraordinary gift of observation of two scientists whose participation confers a particular significance to this gathering there was discovered, thirty years ago, a substance existing in the central nervous system and in the intestine which, though being different from ACh and H (the rôle of 5-HT was not so much as anticipated at that time), produced contractions in the guinea pig ileum and reduced blood pressure in the rabbit. One or two years later this substance was given the name »substance P«. The fact that brain and intestine contain identical or at least similar agents of a polypeptidic character acting like biogenic amines which have a much smaller molecule has brought a fundamentally new concept about factors which can fulfill physiological functions in the central nervous system. Now, after oxytocin, vasopressin and several other centrally acting polypeptides have been found, this concept does not seem to be anything unusual, but thirty years ago it had been a striking novelty.

Von Euler and Gaddum, the discoverers of SP, who also have an outstanding merit for our knowledge about other biogenic amines, have ever since its discovery promoted the studies on SP, but in due course a number of other experimenters also became interested in this field. Almost all of them, it is a pleasure to say, are present here.

At this Symposium we have two basic problems before us: the action of SP in the central nervous system, and its action in the digestive tract. By the way, it is an interesting question in itself why almost every biologically active substance occurring in the digestive tract also occurs in the central nervous system. However, to turn back to the main issue, it is, of course, much easier to study the peripheral effects of SP than its central ones, on the first place because the particular regions of the brain concerned are difficult to approach, and secondly because the responses are complex, and frequently reflect more than one single mechanism.

But the growing importance of central phenomena requires more study of the central effects, and a more favourable balance between papers dealing with peripheral, and such dealing with central effects has been reached at this Symposium.

The remaining important tasks in the SP field to be dealt with are the obtention of more precise knowledge about its distribution, the

finding of specific antagonists, and, possibly, a specific enzyme causing its inactivation. I hope that some of the papers presented here will, in part at least, give answers to these questions and others.

As late as a month ago there seemed to be one problem of great importance — we wanted to put it before this Symposium and even entered it into the programme — namely the question about a standard unit for SP. But an extremely interesting circumstance has made this item unnecessary. Most recently a group of Swiss workers from the Sandoz research laboratories have succeeded in obtaining a SP apparently of absolute purity. This, of course, obviates the need for a conventional standard since absolutely pure SP will serve as a standard in determining the SP-unit.

But this high degree of purity also comprises other implications. It is likely that we shall shortly know the chemical constitution of SP, and it will probably be possible soon to synthesize the compound. So we can tell that we find ourselves at the end of one period in the development of the SP problem, and at the beginning of a new one. The imminent unraveling of the constitution and the possibility to work with a uniform product free from all impurities necessarily imposes the need for a revision of practically all experimental work performed with products obtained by earlier methods of preparation. This, however, does not impair the importance of the papers to be presented at this Symposium because it is very probable that several of the present concepts about the rôles of SP are correct and their validity will remain unchanged.

It will be especially interesting, and the availability of pure preparations will enable us to do so, to elucidate the rôle of impurities masking the real effects of SP. With existing methods it is already possible to see that impurities exert significant component effects. A paper from my laboratory which will be presented at one of the subsequent meetings will show how great the influence of adenosine 5'-monophosphate, for instance, can be with respect to central effects.

One of the concepts mentioned before, which certainly is correct, is that of SP being a transmitting factor in sensory pathways. But, on the other hand, since the distribution of SP has been very thoroughly studied, and its occurence in various parts of the central nervous system established, it seems unlikely that such a substance may be limited to sensory transmission alone. It would rather seem that the rôle of a transmitting factor can be taken up by SP in other mechanisms of the central nervous system too, as it has been found for the biogenic amines. The practically limitless number of possibilities for the action of already known transmitting substances in the individual synapses of the central nervous system shall by no means be modified with the addition of one more transmitter. And, for the rest, we cannot know whether one or more new transmitters of low or high molecular weight will not be found next.

Now, I find, the time has come to say that this assembly should eventually bestow a new designation, a real name to SP. This, of course, will be the exclusive privilege of the discoverers, professors von Euler and Gaddum.

Finally I want to say a few words more. This is the first Symposium dedicated exclusively to SP and, therefore, this gathering may contribute more than any of those held earlier about polypeptides affecting smooth muscle to the efforts of coordinating our investigations, obtaining better collaboration and more extended exchange of experiences. Small Symposia treating one single topic have great advantages for the development of the topic in question, thus we may expect the same from the present Symposium.

Every physiological and pharmacological analysis, in the last consequence, endeavours to make direct or indirect contributions to the advancement of therapy and not only to extend theoretical knowledge. I therefore also wish to express the hope that thorough understanding of the physiology and pharmacology of SP will surely serve for the benefit of ailing mankind.

ACKNOWLEDGMENT. — I am indebted to Dr. Seid Huković who asted as a secretary to the Symposium for his invaluable assistance.

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J. H. GADDUM

THE ESTIMATION OF SUBSTANCE P IN TISSUE EXTRACTS

SP was first recognized in experiments in which ACh was assayed by its action on the blood pressure and the intestine of rabbits. The effect of ACh on these tissues was abolished by atropine, but certain tissue extracts were still effective after atropine and these effects were attributed to SP (Euler and Gaddum, 1931).

Our knowledge of SP still depends on bioassays, but these assays are open to criticism. ACh can be estimated by a number of different sensitive and specific methods and the results are often found to agree with one another. This agreement between parallel assays provides strong evidence that the results give the concentration of ACh itself, and do not depend on allied substances such as propionylcholine (Chang and Gaddum, 1933). Similar methods can be used to distinguish adrenaline and noradrenaline from allied substances (Gaddum, Peart and Vogt, 1949), but when SP is assayed by different methods the results do not agree with one another. This must mean that the extracts used for the assays are not pharmacologically pure; their action does not depend only on SP but also on various other interfering substances which alter the response. Many of these interfering substances are now known and their effects can be eliminated, but there is still no really specific method of assay for SP; estimates should be checked by parallel assays and it is not easy to get concordant results.

There are two ways in which methods of estimation, whether biological or biochemical, may be made more specific. The solutions may be purified, or the final test may be modified. Purified preparations of SP from intestine and brain have been shown to have the same relative potency when tested in various different ways, (Amin, Crawford and Gaddum, 1954, Eliasson, Lie and Pernow, 1956) but purification is laborious and involves loss. Much work has, therefore, been done in various laboratories with the object of making the final test more specific by using antagonists to eliminate the effects of known interfering substances.

The best known interfering substances are ACh and H, and assays of SP are generally done in the presence of antagonists for these two substances. The guinea pig's ileum provides a convenient test (Douglas, Feldberg, Paton and Schachter, 1951; Pernow, 1951) and it

has sometimes been assumed that the effects of tissue extracts on this tissue in the presence of atropine and an antihistamine are entirely due to SP. Amin et al. (1954) found that such tests were often complicated by the presence of 5-HT and precautions are, therefore, generally taken to eliminate effects due to this substance. If the tissue is extracted with 20 volumes of acetone, SP can be quantitatively recovered from the insoluble residue. 5-HT and various other interfering substances are found in the acetone, but it is difficult to extract 5-HT completely from tissues with acetone (Sharman, 1961) and reliable assays, therefore, depend on the use of an antagonist for 5-HT in the final test. The guinea pig's ileum may be densitized to 5-HT by adding tryptamine to the bath fluid (Gaddum, 1953a).

This test has provided much interesting information about SP and its distribution (Zetler and Schlosser, 1955). There is no reason to doubt the general picture given by these results, but it would be unwise to accept such estimates without independent evidence that the effect was really due to SP, and the best evidence of this depends on parallel assays, using a set of tests which have been shown to vary independently in their responses to allied drugs.

Recent work in my own laboratory has been based on 4 methods of assay, using tissues which vary widely in their responses to different drugs, but all of which are fairly sensitive to SP. Various preparations of SP and extracts of tissues have been compared with a standard preparation of SP. This was made by Messrs. Hoffmann La Roche, by Pernow's methods, and adopted by U. S. von Euler and J. H. Gaddum in 1959 as a provisional standard containing 75 units per ml.

The four methods depend on rat uterus, guinea pig ileum, hen rectal caecum and gold fish gut. The first three have been used for some years for detecting pharmacologically active polypeptides and for distinguishing between them (Gaddum, 1955). We have spent some time studying the best conditions for using the hen rectal caecum (Cleugh, Gaddum, Holton and Leach, 1961). This was first used by Barsoum and Gaddum (1935) and was shown by Pernow and Rocha e Silva (1955) to be sensitive to SP. It is also sensitive to ACh, H, 5-HT, and adrenaline, but the effects of these can be diminished by suitable antagonists. Tissue extracts normally contain sufficient ATP and other adenosine derivatives to interfere with the assay by inhibiting the rectal caecum.

Laszlo (1960) has described a method in which adenosine monophosphate is inactivated by an enzyme, but this enzyme does not destroy ATP which may also be present. We have found that these effects can be eliminated by specific tachyphylaxis. If a high concentration of two of these compounds is maintained by adding adenosine (or adenosine monophosphate) and ATP (100 µg of each) to a 4 ml bath whenever it is refilled, the interfering effects of these two substances and also of adenosine diphosphate are usually eliminated.

These adenosine compounds may also interfere with assays on guinea pig ileum, but this effect is less obvious.

The search for a sensitive test for SP has also led to the use of gold fish intestine in a microbath. For many purposes it is convenient to use the method of superfusion (Gaddum, 1953b) in which a piece of plain muscle is suspended in air and suitable salt solution allowed to run over its surface. At intervals the flow is stopped and a drug solution is applied to the surface of the muscle in a volume of 0.25 ml or more. For some purposes it is better to use a microbath (Gaddum and Stephenson, 1958), in which a small piece of muscle is bathed in 0.05 ml of salt solution or less. In the apparatus now used (Gaddum and Szerb, 1961) the bath is a horizontal circular hole (diam. 2 mm.) in a block of perspex. Salt solution runs into a vertical well (diam. 5 mm.), through a small hole into one end of the bath, and then through the bath and down a vertical face at the other end. A length of about 8 mm. of gold fish intestine is suspended between threads in the bath and its movements are amplified about 100—500 times and recorded with a pen writer. This preparation contracts in the presence of SP in a low concentration and can be used to detect quantities of the order of 1—5 milliunits.

It is also sensitive to various other substances present in tissue extracts, but the effects of many of these can be eliminated with antagonists. ACh is antagonized by hyoscine; 5-HT is antagonized by various derivatives of lysergic acid of which methylsergide (UML 491, deseryl, 1-methyl lysergic acid butanolamide) has been found particularly satisfactory, and the inhibitory effects of adrenaline and noradrenaline are antagonized by D. C. I. (dichloroisoproterenol, 3,4-dichlorophenyl-isopropylaminoethanol). ATP causes a contraction, but interference from this source can be avoided by maintaining a constant concentration of ATP in the bath fluid. The preparation is remarkably insensitive to H even without antihistamines.

By such methods all these different kinds of plain muscle — the rat uterus, the guinea pig ileum, the hen rectal caecum and the gold fish intestine can be made insensitive to most of the known pharmacologically active substances in tissues, but there are no known antagonists for the polypeptides and several of these may be present in biological fluids, including not only SP, but also bradykinin, angiotensin, oxytocin and vasopressin. All four muscle preparations are about equally sensitive to SP. The rat uterus is unspecific, being also very sensitive to bradykinin, angiotensin, and oxytocin. The guinea pig's ileum is quite sensitive to bradykinin and angiotensin, though less so than the rat uterus. It is insensitive to oxytocin and vasopressin. The fowl rectal caecum and the gold fish gut are both comparatively insensitive to these other polypeptides and should, therefore, provide specific tests for SP, provided that the effects of ACh, H, 5-HT, catecholamines and adenosine compounds are suppressed by suitable antagonists and provided that they are not affected by other unknown sub-

stances present in the solutions being tested. Experience has shown, however, that the situation is not so simple as was first supposed. When these four tests were applied in parallel to tissue extracts, the results showed discrepancies large enough to suggest the presence of unknown active substances in the extracts.

TABLE I

ASSAYS OF FIVE PREPARATIONS OF SP UNITS PER MG

	Rat Uterus	Guinea pig Ileum	Hen Rect. Caecum	Gold Fish Gut
Standard	75	75	75	75
B	110	37	28	—
D	180	340	375	—
P_3B	31	42	37	30
P_4B	11	35	57	75

Table I shows the results of assays in which four different preparations of SP were compared with the standard. Preparations B and D were obtained by Messrs. Hoffmann La Roche from the standard itself by the method described by Pernow (1953) in which SP is eluted from a column of alumina with methanol diluted with increasing quantities of water. The results show that this standard preparation contains more than one pharmacologically active substance since both of the fractions obtained from it were qualitatively different from it. If this standard preparation was pharmacologically pure the results of the parallel assays of the activity of both fractions would have agreed within the error of the tests. The tests were repeated and it was clear that this was not so. For example, the estimates of the activity of preparation D on rat uterus were 188, 188 and 163, and the corresponding results on guinea pig ileum were 288, 349, 352 and 375. Fraction D was much the most active of these fractions and the simplest assumption is that it contained most of the SP, while fraction B contained some other active substance with relatively more action on the rat uterus. This might be one of the other active polypeptides such as bradykinin, but there is, at present, no evidence of this. The preparation P_3B was made at Babraham by similar methods. There was no very definite difference between the results of the different assays, so that it would be reasonable to conclude that this preparation was similar to the standard. In the case of P_4B, however, the assay on hen rectal caecum gave much higher results than the assay on rat uterus. If the explanation given above to account for the results with preparations B and D is correct, then P_4B is particularly pure from the pharmacological point of view. It contains even less of the substance acting on the rat uterus than preparation D.

The interesting thing about these results is that they were all obtained with active preparations of SP. The preparations used in experiments with this substance have often contained between 5 and 15 units per mg and there is no reason to suppose that they were more nearly pure pharmacologically than the much more active preparations used here. The preparation made by Franz, Boissonnas and Stürmer (1961) contained 30,000—35,000 units per mg. This was presumably pure SP. Most other preparations obtained so far contain pharmacologically active impurities. On the other hand, it is important to remember that the results of experiments of this kind depend on the method of assay used. Most people use guinea pig ileum; if they had used the rat uterus exclusively they might have isolated a different polypeptide and claimed that this was pure SP.

These results have led to the conclusion that the effect of these preparations on the rat's uterus are partly due to an impurity, but we have made no serious effort to find out what this impurity is. We abandoned the use of the rat's uterus and used the other three tests in the hope that they would give concordant results. The estimates given in Table I by these three tests are reasonably concordant; the discrepancies may be due to experimental error. It is possible that any of these methods might be used to estimate the amount of SP in intestine, but there is evidence of some other substance in extracts of guinea pig's brain which complicates the results. J. Cleugh and V. P. Whittaker have been studying the distribution of SP in homogenates of guinea pig's brain after centrifuging.. The brains were homogenized in isotonic (0.32 M) sucrose solutions and tested in guinea pig ileum and hen rectal caecum in the presence of antagonists for known substances. When these homogenates were tested directly they had little activity, but they became active when they were made acid (pH 4) and heated at 100° for 10 min. and then neutralized and tested. This treatment has been shown to liberate ACh in similar circumstances by breaking up the particles in which it is contained and the results suggested that SP was contained in similar particles. When these homogenates were centrifuged most of the activity was found in the »mitochondrial« fraction (P_2) (precipitated at 450,000 g min. but not at 10,000 g min.). This confirms the observations of Lembeck (1960). This fraction was then separated in a density gradient formed by sucrose solutions of different strengths. The activity was mostly found in layer B (between 0.8 M and 1.2 M sucrose). This fraction has been shown to contain the largest amounts of ACh and cholineacetylase (Hebb and Whittaker, 1958), noradrenaline (Chruschiel, 1960) and 5-HT (Whittaker, 1959). Electron micrographs by Gray and Whittaker (1961) indicated that it consists largely of pinched off nerve endings containing synaptic vesicles. The experiments on the distribution of SP have thus led to the conclusion that it is present in the same fraction as substances known (or suspected) to be associated with chemical transmission. This lends support to the theory that it has some func-

tion in the central nervous system, but, unfortunately, the evidence that the substance estimated in these tests was SP itself is not very satisfactory. In some experiments the estimates obtained with the hen rectal caecum agreed well with the estimates obtained with the guinea pig ileum but in other experiments they were larger.

Experiments with gold fish gut revealed the presence of another substance in these extracts which was highly active on this tissue and overshadowed the effects of all the other substances. Since this substance is particularly active on gold fish gut, it was suggested, as a joke, that it should be called aureopiscin and no other name has been suggested so far. This substance is active even in doses corresponding to 10 μg of tissue. It is stable to boiling at pH 2.5 to 9.5 for 10 min. It is present in the supernatant of brain extracts after centrifuging. An extract of guinea pig liver had a similar action. There is clearly much more work to be done on this substance which is interfering with the assay of SP on gold fish gut. It may well turn out to be some substance which is already known to be present in the body, but the gold fish provides a sensitive test for it.

Summary

Four different methods for the assay of SP in tissue extracts have been compared with one another and found to give discordant results, which must be due to the presence of pharmacologically active impurities. This was true, even when the preparation tested was fairly active (75 units per mg). Some of the actions attributed to SP are probably due to these impurities. A method depending on the intestine of the gold fish (Carassius auratus) will detect small quantities of SP, because it can be used in a small bath (0.05 ml), but it is also sensitive to some unknown substance in tissue extracts. It will be necessary to remove this substance from extracts before this method can be used for the assay of SP.

ODREĐIVANJE SP U EKSTRAKTIMA TKIVA

Četiri različite metode za određivanje SP u ekstraktima tkiva uspoređene su međusobno. Rezultati se ne slažu, što mora da je posljedica prisutnosti farmakološki aktivnih onečišćenja. Ovo je zapaženo i prilikom ispitivanja prilično aktivnih preparata (75 jed./mg). Spomenuta onečišćenja, vjerojatno, izazivaju neke od efekata koji se pripisuju djelovanju SP. Pomoću metode s crijevom zlatne ribice (Carassius auratus) mogu se otkriti vrlo male količine SP, jer se određivanje može izvesti u malom kupatilu (0.05 ml), no ona je, istovremeno, osjetljiva na neku nepoznatu supstanciju u ekstraktima tkiva. Prije nego što ova metoda bude upotrebljiva za određivanje SP, treba ukloniti tu supstanciju iz ekstrakata.

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ANU BiH АНУ БиХ

DISCUSSION

EULER: Did you extract the layers after gradient centrifugation or remove the sucrose before assay of the SP effect?

GADDUM: In the experiments I have described, the extracts were made acid and boiled to liberate the SP, but they were not extracted. The dilution was such that the sucrose did not affect the result.

LEMBECK: Do you have to dilute your tissue extracts to the same salt concentration as the Tyrode solution (1:2) when you test them on the gold fish gut?

GADDUM: Yes, the gold fish gut is sensitive to changes of tonicity. The salt concentration of the extracts was adjusted by calculation, so that it should have been the same as that of the bath fluid.

ZETLER: Are these two new biologically active substances contaminating SP destroyed by trypsin or chymotrypsin? Were there also active substances migrating to the anodic side during paper electrophoresis?

GADDUM: I have no evidence of the effects of these enzymes on the substance which is particularly active on rat uterus. They had no effect at all on the substance which is particularly active on gold-fish gut. There was some evidence suggesting that this substance migrates towards the anode at pH 3.4, but further experiments are needed.

HAEFELY: I was very interested to hear from professor Gaddum that he also failed to find a liberation of SP in the *nucleus gracilis*. We did the same experiments using the cats and the dogs as experimental animals and the tissue perfusion technique, I had the chance to see in the institute of professor Gaddum. The needle was put in the *nucleus gracilis* and the

tractus gracilis and was stimulated electrically. Before stimulation there was a weak activity measured on the guinea pig ileum, possibly due to a substance other than SP. After stimulation we failed to demonstrate the change in activity of the fluid coming out the nucleus.

PERNOW: In order to see whether the biological effects obtained by the different extracts are really due to SP we use the following tests.

(1) The specific tachyphylaxis as described by Gaddum and which works very well.

(2) Adsorbtion on aluminium oxide columns and elution with methanol and water.

(3) Inactivation by chymotrypsin is even more active in this respect. but trypsin is preferable since Rocha e Silva and I could show that trypsin inactivates SP but not bradykinin.

ANU ВіН АНУ БиХ

S. HUKOVIĆ, R. KOŠAK AND P. STERN

PARALLEL CENTRAL EFFECTS OF 5-ADENYLIC ACID AND SUBSTANCE P PREPARATIONS OF VARIOUS POTENCIES

Powders obtained in isolation of SP show widely different activities when tested on different organs from different animal species. Such behaviour indicates the presence of substances interfering with the stimulating effects of the active principle. v. Euler and Gaddum (1931) in their first paper on SP suspected ATP-like admixtures to be responsible for the depressive effects of some preparations. In unpublished experiments carried out in order to concentrate SP we found that some fractions exerted a marked depressive effect on the guinea pig ileum; we also observed that certain preparations containing SP, on addition to the bath fluid, first caused a dilatation of the organ, and afterwards only the expected contraction. Laszlo (1960) suggested 5-adenylic acid (5-AA) to be one of the ATP-type impurities present in raw SP. By the action of adenosine monophosphatase (AMP-ase) this acid is converted to 5-inosinic acid, a product completely devoid of any action on guinea pig ileum. Two of us (Stern and Huković, 1960) noticed that strongly active preparations (270 units SP per mg) lack some of the central effects described by Zetler (1956), who worked with weak preparations of 8 U./mg potency. The concentrated preparation did not inhibit strychnine convulsions, nor did it lengthen hexobarbital narcosis, but it antagonized the analgetic action of morphine in mice even in very small doses (1 unit per gram body weight). This antagonism is fully in agreement with Zetler's (1956) findings. Lembeck (1953) forwarded the hypothesis that SP represents the transmitter substance of the sensory neurons. It is entirely acceptable that a substance concerned with sensory transmission should antagonize inhibitors of sensitivity.

Since 5-AA goes along with SP in organ extracts it was necessary to establish the central effects of the former in order to hold them apart from those of the latter.

Method

In these experiments we used mice of both sexes, weighing 18 g on the average. Controls were set up in all experiments by injecting a number of animals with the same volume of saline or other solvent

used for preparing the solutions of SP injected to experimental animals. The injections in both experimental and control animals were applied on identical places at the same time intervals. Injections of strychnine were made according to Orlow, Williams and Pfeiffer (1949): strychnine nitrate (36 μg/ml) in saline was injected into the dorsal vein of the tail at a rate of 0.05 ml/0.1 min. till the appearance of tonic convulsions. Hexobarbital (sodium salt) (2.20 μg/ml) was applied in the same manner, at the same rate, in a total dose of 55 μg per g body weight. The duration of narcosis was measured from the end of the injection until the first signs of wake-up (righting reflex). Solutions of the sodium salt of 5-AA (10 mg/ml) were freshly prepared and injected i. p. in doses of 0.20 mg per g body weight 15 min. prior to strychnine, hexobarbital or to the production of pain by application of heat. The degree of pain was rated according to the method by Woolf and McDonald (1944). Morphine hydrochloride solution (0.24 mg/ml) in a dose of 6 μg/g was injected s. c. 30 min. before 5-AA and its analgetic effect estimated 45 minutes later. AMP-ase was prepared from rabbit muscle by the procedure of Schmidt (1928). The enzyme solution was neutralized with 10 per cent acetic acid, filtered, and stored under toluene. Enzymatic activity was determined by measuring the volume of ammonia evolved from 5-AA after incubation for 2 hours at 30°C and pH 5.9 (phosphate buffer) in a van Slyke—Cullen volumetric apparatus. The enzyme after neutralization contained an extremely small amount of ammonia. It liberated from an SP preparation of 25 U./mg potency practically the same amount of ammonia as from the same weight of 5-AA.

Statistical evaluation was carried out according to Fisher (1948) and the statistical significance between mean values judged by Student's t-test. Standard error of the mean is denoted by SE in the Tables.

Results

The first series of experiments was intended to show the influence of 5-AA on the action of strychnine, hexobarbital and morphine (Table I).

The dose of 662.50 ± 29.22 μg/g strychnine nitrate was absolutely lethal, after heavy convulsions, in the controls. Administration of 5-AA 15 min. prior to the strychnine salt protected the animals from convulsions and raised the lethal dose to 914.70 ± 47.31 μg/g. The difference is significant, $p<0.01$. At a longer time-interval between 5-AA and strychnine, e. g. more than 30 min., there was no more protection against convulsions.

Controls receiving hexobarbital alone sleep 10.2 min. in the average (SE ± 2.63 min.). Sleeping time is doubled when 5-AA is given prior to hexobarbital (20.76 ± 3.155 min.). The difference is significant, $p < 0.01$.

Controls react to pain 0.20 ± 0.131 min. after application of heat. Animals previously injected with 5-AA react only after 0.38 ± 0.0268

min. The difference is significant, $p < 0.01$, thus, 5-AA acts analgetically. When morphine is administered 45 min. prior to application of heat the animals react after 0.27 ± 0.0155 min. With 5-AA the corresponding time lag is 0.74 ± 0.0393. The difference is significant, $p < 0.05$, 5-AA potentiates the effect of morphine.

TABLE I

THE PROTECTIVE EFFECT OF 5-ADENYLIC ACID TOWARD CONVULSIONS PRODUCED BY STRYCHNINE

Previous administration	Strychnine i. v. µg/kg	S E	n	t	p
0.90% NaCl, i. p.	662.50	29.22	12	—	—
5-Adenylic Acid, 0.20 mg/g i. p.	914.70	47.31	12	3.552	< 0.01

THE PROLONGATION OF EVIPAN NARCOSIS BY 5-ADENYLIC ACID. EVIPAN SODIUM 55 µg/g i. v. IN MICE

Previous administration	Duration of narcosis in minutes	S E	n	t	p
0.90% NaCl, i. p.	10.02	2.625	10	—	—
5-Adenylic Acid, 0.20 mg/g i. p.	20.76	3.155	10	3.153	< 0.01

THE ANALGETIC OF 5-ADENYLIC ACID AND POTENTIATION OF ANALGETIC EFFECT OF MORPHINE

Previous administration	Time of reaction in min.	S E	n	t	p
0.90% NaCl, i. p.	0.20	0.0131	12	—	—
5-Adenylic Acid, 0.20 mg/g i. p.	0.38	0.0268	12	5.789	< 0,01
5-Adenylic Acid and Morphine, 6 µg/g	0.74	0.0393	12	2.448	< 0.05

The next series of experiments was carried out with SP. The preparations had 25 U./mg potency and were previously incubated with AMP-ase. The same drugs were tested as before. Controls were injected with buffered enzyme solution alone. The results are shown in Table II.

The controls exhibited strychnine convulsions and died of 542.90 ± ± 20.92 µg/g strychnine salt. With incubated SP solutions the animals were protected against convulsions and died only after 888.30 ± 80.91 µg/g strychnine salt. The difference is significant, $p < 0.01$. Sleeping time after hexobarbital in controls was 14.14±4.466 min. With incubated SP solution the sleeping time was increased to 23.14±4.734, but the difference is not significant at $p < 0.30$. After morphine alone animals reacted to applied heat after 0.46 ± 0.0477 min. With incubated SP solution injected 30 min. after morphine this interval was reduced to 0.27 ± 0.0277, which is almost the same as in controls after saline and buffered enzyme solution alone (0.30±0.0216 min.). The difference in reaction interval between controls and experimental animals is significant at $p < 0.01$.

TABLE II

THE PROTECTIVE EFFECT OF SUBSTANCE P TOWARD CONVULSIONS PRODUCED BY STRYCHNINE NITRATE i. v. ON MICE

Previous administration	Strychnine i. v. μg/kg	S E	n	t	p
0.90% NaCl, i. p.	542.90	20.92	10	—	—
Incubated SP, 10 μ/g i. p.	888.30	84.91	10	3.590	< 0.01

THE PROLONGATION OF EVIPAN NARCOSIS BY SUBSTANCE P. EVIPAN SODIUM 55 μ/g i. v. IN MICE

Previous administration	Duration of narcosis in minutes	S E	n	t	p
0.90% NaCl, i. p.	14.14	4.466	12	—	—
Incubated SP, 10 μ/g i. p.	23.04	4.734	12	1.358	< 0.30

THE INHIBITION OF ANALGETIC EFFECT OF MORPHINE BY SUBSTANCE P. MORPHINE HYDROCHLORIDE s. c. 6 μg/g OF MICE

Previous administration	Time of reaction in min.	S E	n	t	p
0.90% NaCl, i. p.	0.30	0.0216	12	—	—
Morphine, s. c.	0.46	0.0447	12	5.678	< 0.01
Morphine and Incubated SP, i. p.	0.27	0.0274	12	5.987	< 0.01

Injection of a large dose (0.0125 ml/g i. p.) of SP resulted in hypnotic, toxic and lethal effects. Unincubated SP powder of 25 U./mg potency was lethal in a dose of 40 U./g body weight. The same dose of incubated SP of identical potency killed 11 out of 12 animals. After incubation of the same powder with chymotrypsin the same dose, again, killed 7 out of 8 animals. Both unincubated and incubated powder still had a lethal effect if given in a dose of 30 U./g. With 20 U./mg only toxic effects in form of narcosis were observable. With further reduction of the dose other effects became manifest. With 14 U./g one can well see muscular pareses and weakness of the skeletal muscles. The toxic effects of SP start with paresis of the hind legs, difficulties in respiration, and, in some animals, rolling round their longitudinal axis. Cessation of symptoms and awakening occurs suddenly.

Discussion

Two of us (Stern and Huković, 1960) have already reported earlier that an SP powder of 16.3 U./mg potency inhibited strychnine convulsion, lengthened hexobarbital sleeping time, and antagonized morphine analgesia. In the present work we obtained the same effects with a more concentrated preparation of 25 U./mg, in which polluting 5-AA was destroyed by incubation with AMP-ase. 5-AA, itself, proved in our experiments to inhibit strychnine convulsions and lengthen hexobarbital narcosis. This substance, however, potentiated the analgetic action of morphine, and exhibited moreover some analgetic action of its own. The

difference in action between both incubated and unincubated SP preparations on the one hand, and 5-AA on the other lies in the effect on the central action of morphine. In trying a high-potency SP preparation (270 U./mg) we found that it did not protect the animals from strychnine convulsions, nor lengthen hexobarbital narcosis, but it antagonized morphine analgesia, in small doses*). Thus all SP preparations examined by us had one feature in common, namely the antagonism to morphine analgesia (Table III). The depression of strychnine convulsions and the lengthening of hexobarbital narcosis observed with raw SP preparations are probably to be attributed to the action of 5-AA. The antagonism to morphine, however, is a characteristic feature of SP, and 5-AA acts here in the opposite sense. These results show that in addition to previously established inhibition of SP-effects on the guinea pig ileum 5-AA also interferes with central effects of SP. It will, therefore be interesting to study, by the same methods, a high-potency SP preparation (30,000 U./mg) obtained recently by Franz, Boissonnas and Stürmer (1961) which appears to be the pure polypeptide, in order to establish definitely the true central effects of SP.

TABLE III

COMPARISON OF THE CENTRAL EFFECTS OF SP AND 5-ADENYLIC ACID AFTER STRYCHNINE, HEXOBARBITAL AND MORPHINE ON MICE

Previous administration	Strychnine convulsions	Hexobarbital narcosis	Morphine analgesia
Powders of SP 25 μ/mg Incubated with AMP-ase 10 μ/g i. p.	Inhibition $p < 0.01$	Prolongation $p < 0.01$	Inhibition $p < 0.05$
Powders of SP 16.2 μ/mg Nonincubated. 10 μ/g i. p.	Inhibition $p < 0.01$	Prolongation $p < 0.30$	Inhibition $p < 0.05$
Powders of SP 270 μ/mg Nonincubated 10 μ/g	Stimulation $p < 0.20$	0	0
Powders of SP 270 μ/mg Nonincubated 1 μ/g	0	0	Inhibition $p < 0.05$
5-Adenylic Acid 0.20 mg/g i. p.	Inhibition $p < 0.01$	Prolongation $p < 0.01$	Stimulation $p < 0.01$

Our results confirm the transmitter rôle of SP in sensory neurons proposed by Lembeck (1956). Antagonism to analgetic action in a factor supposed to facilitate the transmission of sensory impulses is an obvious property. This idea is also suggested by findings of Holton (1960), Serafimov (1958) and Stern and Kocić-Mitrović (1958). The two latter authors supplied some evidence to the above statement by showing the influence of light and dark on the amount of SP in the retina.

The lethal effect, toxicity, and hypnotic action of the 25-U./mg SP-powder, unincubated as well as incubated with AMP-ase and chy-

*) It must, however, be pointed out that morphine antagonism could be observed only with small doses, 1 U. per g body weight. Doses of 10 U./g failed to antagonize morphine and even potentiated strychnine convulsions.

motrypsin, point to the possible existence of further impurities. In this respect it will be particularly interesting to examine the type of muscular pareses by which the toxic symptoms start. One may ask first whether they are a consequence of peripheral or central SP effects.

Finally, to turn back to 5-AA, it is important to mention that the action of this substance is strongest within the first 15 min., and diminishes during the next 30 min. Such time-action relationships have also been found for SP by v. Euler and Pernow (1954) and Zetler (1959), and this coincidence affords additional support to the inference that the central effects of SP are in part due to 5-AA. So once more one can say that the central effects peculiar to SP shall only be known when large amounts of pure SP, free from interfering admixtures, will be available for further study.

Summary

5-Adenylic acid inhibits strychnine convulsions, lenghthens hexobarbital sleeping time, and stimulates the analgetic action of morphine.

SP-preparations, up to 25 U./mg potency, act in the same way on strychnine convulsions and hexobarbital narcosis, but antagonize morphine analgesia. These effects remain unchanged after incubation with AMP-ase carried out in order to destroy polluting 5-Adenylic acid.

The difference in action between SP-preparations containing 5-Adenylic acid and authentic 5-Adenylic acid lies in the effect on morphine analgesia alone.

Toxic effects observed with SP-preparations up to 25 U./mg potency are probably due to further impurities.

In order to study the true central effects of SP in detail very pure preparations with high SP potency are needed.

PARALELNI CENTRALNI EFEKTI 5-ADENILNE KISELINE I SP-PREPARATA RAZLIČITE JAKOSTI

5-adenilna kiselina djeluje inhibitivno na strihninske grčeve, produžuje heksobarbitalsku narkozu i stimulira analgetsko djelovanje morfina.

Preparati SP do jakosti od 25 jed./mg djeluju jednako na strihninske grčeve i heksobarbitalsku narkozu, ali na analgetsko djelovanje morfina reaguju antagonistički. Ovi efekti ostaju bez promjene poslije inkubacije s adenozin-monofosfatazom u cilju inaktivacije primiješane 5-adenilne kiseline.

Različito djelovanje preparata SP s primjesom 5-adenilne kiseline i same adenilne kiseline očituje se jedino u odnosu na analgetsko djelovanje morfina.

Toksički efekti zapaženi kod preparata SP do jakosti od 25 jed./mg su, vjerojatno, posljedice daljih onečišćenja.

Za proučavanje pravih centralnih efekata SP u pojedinostima potrebni su veoma čisti preparati s velikom jakošću.

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DISCUSSION

ZETLER: Did you try to destroy the strychnine-antagonistic and the hexobarbital-synergistic activity of SP-powder by proteolytic enzymes?

HUKOVIĆ: We only destroyed the 5-adenylic acid by monoaminophosphatase, using the method described by Laszlo. We did not perform experiments with powders of SP previously incubated with proteolytic enzymes.

PERNOW: If Lembeck is right when he thinks that SP is a transmitter of the first sensory neurons, and I think that is an attractive hypothesis, then it is convincing to hear, that with the more purified SP preparations there is no prolongation of the Evipan narcosis, which has been shown to be obtained with the crude preparations. If the SP is supposed to facilitate the transmission of sensory nerves, then rather a shortening of the narcosis time would be expected.

Have you tried to separate the factor in the crude SP preparation responsible for the prolongation on the narcosis from the real SP factor?

HUKOVIĆ: In these experiments we have used crude SP. As regards separating of different factors from crude SP, we started separating them, but we still did not inject them.

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K. UMRATH

THE RELATION OF SUBSTANCES P TO NEUROTRANSMITTER SUBSTANCES

Some years ago I came to conceive the opinion, that an aqueous extract of dorsal roots contains a substance, which is a compound of a polypeptide with the transmitter substance of sensory nerves, and which has a high P-activity on the guinea pig ileum. With an enzyme, that can be obtained from nervous tissue, this compound can be split in an astonishingly short time into the polypeptide and the transmitter substance of sensory nerves. By this procedure the capillary dilatatory and reddening action on the denervated rabbit ear is not altered, but the P-activity on the guinea pig ileum is reduced to a low degree, which is about the same as that of an aqueous extract of ventral roots (Umrath, 1953, 1956). I will call this enzyme »Pease«. Since some sensory nerves, like the optic and the stato-acoustic nerve, have as low P-activities as the ventral roots, I tried to solve the question, whether their transmitter substance is different from that of the fibres in the dorsal roots.

In a series of rabbits one ear was denervated and the other left as a control. As it is known from previous work, the sensitivity of the denervated ear to extracts of dorsal roots is increased, but its sensitivity to ACh, H, ATP and to extracts of ventral roots is reduced (Hellauer and Umrath, 1948; Florey and McLennan, 1955). Now it turned out, that after denervation, at least in the first three weeks, the sensitivity for extracts of the optic nerve is decreased. So it is to be concluded, that the optic nerve possesses a transmitter substance different from that of the dorsal roots. I will call the transmitter substance of the optic nerve opticin and that of the dorsal roots dorsin. Only dorsin can be tested on the denervated rabbit ear, but both are effective in the bee-test of Florey (1951). This test uses positive phototactic bees, with cut wings, and with one eye punctured, in a faintly illuminated room. Such a bee takes an irregular course, without prefering the one or the other side. If a small quantity of an extract of dorsal roots or of optic nerve is applied to the punctured eye, the course of the bee deviates to the side of the treated eye, as if this eye were more illuminated than the other. Actually these substances, which enter the punctured eye, are the same as those normally produced in the eye by light.

Using these test methods it is possible to investigate the enzymatic breakdown of these transmitter substances. Working with aqueous extracts of the optic nerve, one finds what one would expect from analogy with ACh and acetylcholinesterase. Opticin is destroyed by an enzyme, opticinase, which is contained in the optic nerve in such a concentration, that by adding unboiled, crushed material from the optic nerve diluted 1:200, the effect of opticin in the bee-test is destroyed in about one hour. One gets nearly the same result with a 1:200 dilution of crushed nervous system from arthropodes or from annelides. If one takes dorsal roots (1:200) as the enzyme, opticin is destroyed in about 2 hours.

For dorsin I had three methods of preparing the solution. (1) Boiling 1 part of dorsal roots in 3 parts of salt solution. (2) Boiling, as before, 1 part of dorsal roots in 3 parts of salt solution and adding, after the decanted extract has cooled »Pease« (1 part of original nervous tissue to 1,000 parts of extract of dorsal roots); in about 10 minutes the action of »Pease« is completed and the extract is subsequently boiled for several minutes in order to destroy the »Pease«. (3) Boiling 1 part of dorsal roots with 3 parts of ethanol, decanting, evaporating the ethanol at 30°C and at reduced pressure, and dissolving the residue in 3 parts of salt solution. The P-activity on the guinea pig ileum of solution 1 is high, those of solutions 2 and 3 are low to an equal degree and nearly as low as the P-activity of extracts of ventral roots or of optic nerve. Only in solutions 2 and 3 dorsin is readily destroyed by dorsinase.

In the bee-test, using crushed dorsal roots (1:200) as the enzyme, the dorsin activity in solutions 2 and 3 is destroyed in 1—2 hours; using nervous system of arthropodes (1:200) the activity in solutions 2 and 3 is also destroyed in 1—2 hours, and, using crushed optic nerve, in 3—6 hours. In contrast to this, in solution 1, using the enzyme from dorsal roots (1:200), the dorsin activity in the bee-test is only destroyed after 4 hours.

In the test on denervated rabbit ear the apparent time intervals required for destruction are 2—3 times longer than in the bee-test. The reason may be that the residual solution after the action of dorsinase has an antagonistic action to dorsin in the bee-test, but a synergistic action to dorsin in the test on the denervated rabbit ear. If one would perceive, in the bee-test, the destruction of $^1/_3$ of the dorsin, and in the test on the denervated rabbit ear the destruction of $^2/_3$ of the dorsin, this would explain the differences in time. In using the test on the denervated rabbit ear one finds the dorsin activity in solution 2 and 3 destroyed by crushed dorsal roots or arthropode nervous system (1:200) in 3—5 hours, but in solution 1 by crushed dorsal roots only in 10—18 hours and by arthropode nervous system, in the same time interval, only in a barely detectable amount.

From these findings I conclude, that free dorsin exists in solutions 2 and 3, that can be destroyed equally well by dorsinase of the ver-

tebrate and of the arthropode nervous system, but that there is a dorsin-polypeptide compound in solution 1, with a high P-activity on the guinea pig ileum, which is not destroyed by dorsinase, so that the dorsin must be split off the polypeptide by »Pease«, before dorsinase can act. As it is known (Umrath, 1953, 1956), it takes much time to form »Pease« from crushed nervous system.

I imagine, that all the transmitter substances are bound to polypeptides, if stored in the nervous elements. The exception in the case of dorsin would be only, that its polypeptide-compound would not be split by boiling in water. In many cases the polypeptide compound is the precursor of the transmitter substance, in other cases it seems to play itself the role of the transmitter substance. According to von Oer (1961) one has to assume an ACh-compound, which is not destroyed by cholinesterase, as the transmitter substance from the epithelium of the cornea to the sensory nerves of the cornea and from secondary sense cells to their sensory nerves. Since we have seen, that the dorsin-polypeptide compound is not destroyed by dorsinase, it is very likely, that an ACh-polypeptide compound, not destroyed by cholinesterase, is the transmitter from the epithelium of the cornea and from secondary sense cells to sensory nerves.

Most work on SP was done with SP from intestine. It is available in crude extracts and in purified preparations. In both forms its P-activity is reduced by »Pease« in a short time to a lower level, which is not further decreased by longer exposure to »Pease«. From this I conclude, that SP from intestine is a polypeptide with a prosthetic group, which can be split off by »Pease«. SP from the intestine is active in the test on denervated rabbit ear, but this activity is not altered by »Pease« and almost not altered by trypsin. With an impure trypsin I could reduce the P-activity of a purified SP from intestine, estimated on the guinea pig ileum, to 2% of the original activity, reducing thereby the activity on the denervated rabbit ear to about 50%. From these findings I conclude, that the prosthetic group is mainly responsible for the activity on the denervated rabbit ear, but this activity is not destroyed by dorsinase, as it is in the case of SP from dorsal roots, the dorsin-polypeptide compound. A more obvious difference is, that SP from intestine has no activity in the bee-test, even at high concentrations of purified preparations, whereas, as already mentioned, SP from dorsal roots, the dorsin-polypeptide compound, has a good activity in the bee-test.

Summary

SP from dorsal roots is supposed to be a polypeptide with a prosthetic group, which is dorsin, the transmitter substance of the sensory nerve fibres in the dorsal roots.

SP from nervous elements not containing dorsin is supposed to be a polypeptide, which before extraction bore the transmitter substance of the neurons as a prosthetic group. Extraction with boiling

ethanol yields a polypeptide without a prosthetic group from all nervous elements, including dorsal root.

SP from intestine is supposed to be a polypeptide with a prosthetic group, different from dorsin, but similar to it in certain respects.

ODNOS SP I NERVNIH TRANSMITORA

Pretpostavlja se da je SP iz dorzalnih korjenova polipeptid s prostetičkom grupacijom. Ova grupacija je identična s dorzinom, transmitorom senzornih vlakana u dorzalnim korjenovima.

SP iz nervnih elemenata što ne sadrže dorzina smatra se polipeptidom, koji je prije ekstrakcije bio vezan s prostetičkom grupacijom identičnom s transmitorom odnosnih neurona. Pri ekstrakciji ključalim etanolom otcjepljuju se prostetičke grupacije svih nervnih elemenata, uključujući i one dorzalnih korjenova. Za SP iz crijeva se pretpostavlja da nosi prostetičku grupu različitu od dorzina, ali po nekim efektima sličnu ovom transmitoru.

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DISCUSSION

LISSAK: May I ask professor Umrath what is his opinion about the role of ACh content of sensory nerves (n. opticus, dorsal roots), whether he accepts the standpoint of O. Loewi and Hellauer, or mine and that of Brecht and Corsten? Have you tested the »Opticine« content of the retina? It is well known that the retina has an exceptionally high ACh content.

UMRATH: The figures for the ACh content of sensory nerves given by Loewi and Hellauer are about the same as those given by you or by Brecht and Corsten. Loewi thought, that such a small ACh content in sensory nerves is insignificant and so Loewi and Hellauer summarized, that sensory nerves contain no ACh. There can be no doubt, that in sensory nerves as a whole small quantities of ACh are present, but the question is, whether every sensory fibre contains a small amount of ACh, or if the total amount of ACh belongs to a small number of cholinergic fibres. An investigation on trophic influences of neurons in the nervous system by me and Hellauer (Deutsche Z. f. Nervenheilkunde, 1951, 165, 409—429) showed, that after sectioning the sciatic nerve, ACh in the central stump is augmented to 132%, calculated on wet weight of nerve, or, as the nerve stump gains on weight, to 161% calculated on even lenght of nerve. After removal of an eye from a rabbit we found the ACh content in the residual optic nerve and in the crossed optic tract to be augmented on the 4th day in 3 experiments to 158%, 161% and 226% calculated on wet weight of nerve. As the afferent, sensory fibres of the optic nerve have their cell-bodies in the retina and therefore degenerate after removal of the eye, our results, as I think, show conclusively, that the ACh in the optic nerve is located in a

small number of efferent, cholinergic fibres. In our experiments the sheath of the optic nerve was not removed and the ACh content of the normal control nerve was 0,5 μg/g.

To account for the ACh content of dorsal roots, there are, in my opinion, enough parasympathetic pathways leaving the spinal cord by the dorsal roots. Their cholinergic preganglionic fibres leave the spinal cord by the dorsal roots and, in the dorsal root ganglia, join the cholinergic ganglionic neurons, which send their axons through the dorsal roots and autonomous nerves to the innervated organs.

We have not yet tested the opticin content of the retina, but. as Dr. Vogt pointed out, the retina has several different neurons and therefore may have several different transmitter substances.

STÜRMER: We agree with Prof. Umrath's statement that SP is not always completely destroyed by incubation with trypsin. Our highly purified SP preparation retained some biological activity after incubation with trypsin for three hours at 25°C.

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H. CASPERS

SOME ACTIONS OF SUBSTANCE P ON THE CEREBRAL CORTEX AND THE BRAIN STEM RETICULAR FORMATION

Since the basic investigation on the properties of SP by von Euler and Gaddum (1931) numerous experiments have provided evidence that the polypeptide, besides its well-known action on smooth muscle organs, exerts a definite influence on several central nervous functions. Among the cerebral effects hitherto described a general decrease of vigilance following a parenteral application of the substance represents a most striking phenomenon which has been demonstrated in various animal species [cf. v. Euler and Pernow (1954, 1956), Zetler (1956, 1959), Stern (1959), Stern and Huković (1958), Stern and Dobrić (1957) et al.]. In spite of the fact that this action of SP implies important problems both from a neurophysiological and a pharmacological point of view, little is known as yet about its fundamental mechanisms. The following experiments were designed to establish whether changes of cortical and reticular activation processes are involved in generating the sedative effect. The results obtained provide a first and, surely, limited insight into the origin of the quieting phenomenon which, as a whole, is probably a very complicated one. With respect to previous findings concerning the correlation between behavioural activity changes of the animal and cortical d. c. deviations (Caspers, 1959, 1961; Caspers and Schulze, 1959) the steady potential of the cerebral cortex (d. c. component) served as the principal indicator of the investigated SP actions. A more detailed account of the general experimental procedures adopted will be given in the following sections of this paper.

Methods

The investigations were performed on non-anaesthetized, freely moving rats with chronically implanted electrodes. The steady potential of the cerebral cortex was led from various motor, sensory and association areas of the intact surface and recorded against a common reference point in the front portion of the muzzle which is sufficiently indifferent. In each of the subsequent figures an upward deflection of the d. c. records indicates a negativity of the active electrode on the

cortical surface, and vice versa. Further details of the d. c. recording techniques adopted have already been published elsewhere [Caspers (1959, 1961); Caspers and Schulze (1959); Caspers and Stern, (1961)]. Apart from the d. c. potential of the cerebral cortex the motor activity of the animal, the conventional EEG and the unit discharges in various parts of the midbrain reticular formation were recorded. In most of the experiments additional records were taken from extrapyramidal motor structures of the brain stem, particularly from the nucleus ruber and its surrounding areas.

Throughout the experiments 3 SP-preparations containing 6.7; 16.5 and 20 units SP per mg were available. As a rule, the (diluted) substance was administered intraperitoneally (i. p.) in amounts ranging from 500 to 10,000 U./kg body weight. According to the results of control experiments the typical effects of SP described in the following sections of this paper are either extinguished or at least greatly reduced by a preceding incubation of the substance with trypsin. This finding indicates that the main effective component of the applied inhomogenous preparations actually represents a polypeptide.

Results

I. **Normal variations of the cortical d. c. potential in the freely moving rat.** — In the freely moving rat the cortical d. c. potential shows considerable fluctuations which are synchronized with behavioural activity changes of the animal. During the transition from wakefulness to sleep, for instance, the base line of the d. c. record shifts to the positive side relative to the mean waking level. An arousal reaction, on the other hand, is invariably associated with a negative displacement of the surface steady potential. In the waking animal similar negative d. c. deflections occur with spontaneous (orienting) movements or can be induced by various sensory stimuli. The extent, voltage and time course of such peripherally evoked d. c. shifts depend on the intensity, on the repetition rate and on the modality of the stimulus applied. Negative deflections of considerable amplitude are particularly released by tactile stimulations of the whiskers and of frontal head areas. In experiments on less tamed rats similar high voltage d. c. deviations to the negative side which correspond to the behavioural attention reaction can be initiated by approaching the animal to a critical distance. As compared with tactile stimuli tone pulses, for instance, produce rather small and often circumscribed d. c. reactions which rapidly adapt. Amplitude and extent of such acoustically evoked shifts, however, tend to increase if conditioned stimuli are employed. According to our present experience such conditioned auditory d. c. reactions are, in addition, less susceptible to adaptation.

Some of the d. c. findings summarized above are illustrated in Fig. 1 which represents an original record of cortical d. c. and a. c. potentials at a low paper speed in an awake and freely moving rat. In general agreement with the results of previous investigations which

have been described in detail elsewhere (Caspers et al.) the tracings indicate that the d. c. component reflects both the spontaneous and the evoked activity changes of the cerebral cortex more precisely than the conventional EEG. In close accordance with the behavioural reactions of the animal voltage, extent and duration of an induced d. c. shift are obviously related to the amount of »information« which a given stimulus contains. As could be concluded from earlier experiments (Caspers and Schulze, in press), the widespread d. c. displacements are mediated, at least preferentially, by the brain stem reticular formation. For these reasons the steady potential of the cerebral cortex can be regarded as a suitable indicator for analyzing the actions of SP on the reticulo-cortical system.

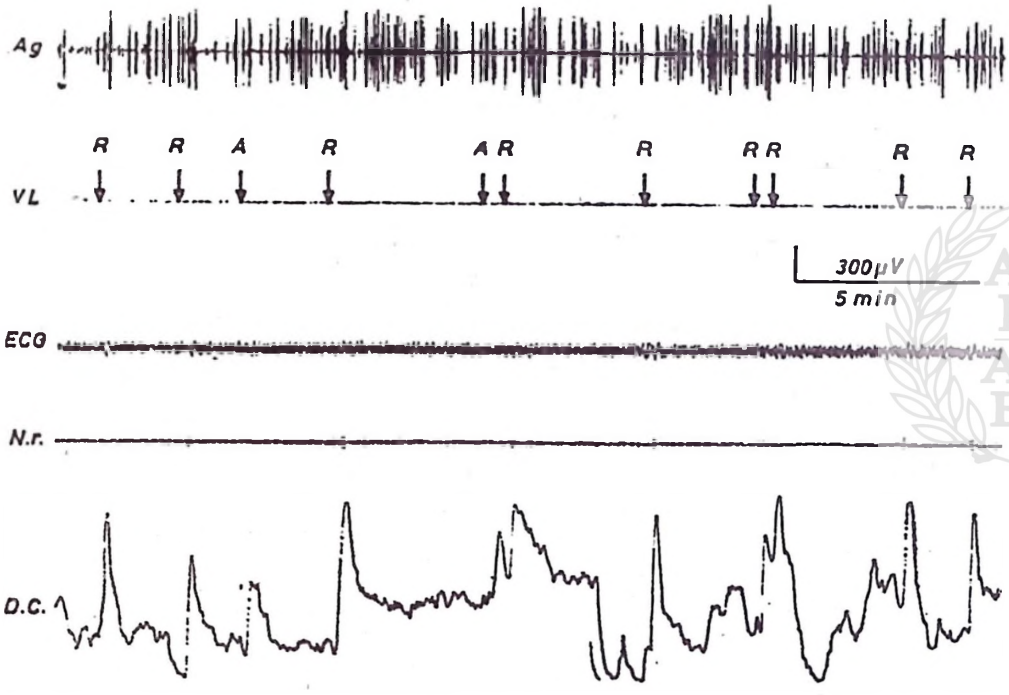


FIG. 1

Simultaneous recordings of the cortical d. c. potential (D. C.), the unit activity of the nucleus ruber (N. r.), the conventional electrocorticogram (ECG) and of the motor activity of the animal (Ag; tracing of a vibration box). The curve N. r. was additionally recorded by an oscilloscope. The moment of stimulation is indicated by arrows on the comparison line (VL). R: Tactile stimulation of the vibrissae. A: Approach by the experimenter. As in the subsequent figures an upward deflection of the d. c. curve indicates a negativity of the active electrode on the cortical surface. In this experiment a subsequent treatment of the animal with 3 mg chlorpromazine effected a complete suppression of the normal d. c. fluctuations thus proving their biological origin.

Some experimental results concerning the fundamental mechanisms possibly involved in generating the d. c. component have already been discussed in previous papers (see Caspers et al.). The available data support the preliminary hypothesis that the d. c. recordings represent a widespread integration of polarization changes in the mass of

apical dendrites and/or other cortical elements which are initiated, for instance, by afferent neuronal impulses or by humoral agents.

II. **The action of SP on the cortical d. c. potential.** — After an i. p. application of 4,000—8,000 U./kg body weight the steady potential of the cerebral cortex shows a distinct positive shift which is usually associated with an increase in voltage and duration of the EEG waves. Apart from quantitative differences these bioelectrical effects, therefore, resemble both the d. c. and the EEG alterations occurring at the onset of natural sleep. The positive displacement of the d. c. component elicited by SP starts after a short latency and reaches a maximum value of 2—4 mV within 10—15 min. after the injection (Fig. 2). The d. c. shift having flattened and finally ceased, the base line remains deviated to the positive side for a variable time and then gradually returns to the initial level.

Monophasically positive d. c. reactions as described above represent a typical SP effect. They particularly occur in either case in which a relatively small amount of SP ranging from about 2,000 to 4,000 U./kg is administered. Higher doses of SP, however, often release multiphasic d. c. deviations. In these experiments the initial positive shift is followed by a rather strong negative d. c. deflection which compensates and sometimes even exceeds the preceding positivity. An example of such a finding is illustrated in Fig. 2. The basic mechanisms of the secondary negative shifts which are due to extracerebral actions of SP will be discussed in a later section of this paper.

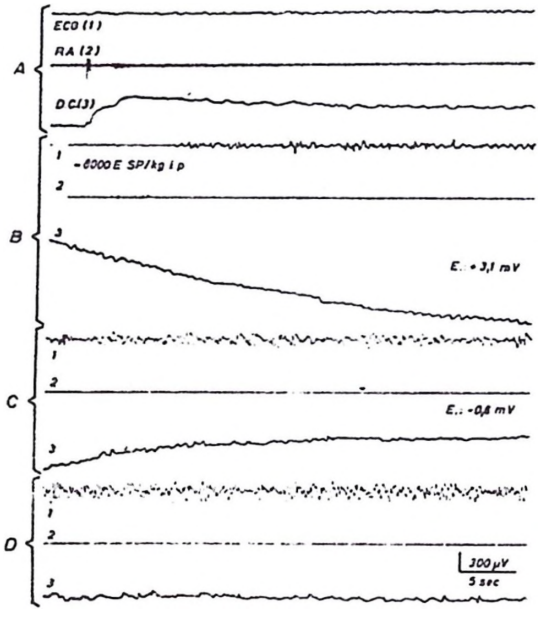


FIG. 2

The action of 8,000 U./kg SP i. p. on the electrocorticogram (ECG; 1), on the activity level of midbrain reticular neurons (R. A.: 2) and on the base line of the cortical steady potential (D. C.; 3). Tracing 2 was simultaneously recorded by an oscilloscope. A: Normal record with a spontaneous activation of the animal at the beginning of the curve. B: Positive shift of the steady potential immediately after the application of SP. C: Secondary negative d. c. shift 4 min. later. The letter E in B and C indicates the final amount of the positive and negative d. c. deviation. D: Stable base line of the d. c. component about 18 min. after the SP application.

The base line deviations of the steady potential which were hitherto discussed are always associated with an extinction or a strong reduction of the spontaneous d. c. fluctuations occurring before the application of SP. This stabilization of the d. c. component corresponds to the behavioural sedative effects elicited by the polypeptide. In addition to the spontaneous shifts the evoked negative d. c. deflections, too, are either abolished or decreased. Fig. 3 demonstrates a temporary extinction of a peripherally induced negative d. c. displacement after the i. p. application of 8,000 U./kg body weight. The various tracings in Fig. 3 furthermore indicate that the suppression of the evoked negative d. c. shifts is closely related to another SP effect. In the freely moving animal an induced rise of the cortical excitation level reflected in an extensive and high voltage d. c. deviation is frequently associated with an activation of the extrapyramidal motor system of the brain (Fig. 3 A 1). This concomitant activation of motor structures is reduced by SP and usually disappears when the evoked d. c. shift reaches a critical minimum (see also Fig. 4). This finding points to a certain causal link between the basic mechanisms responsible for the cortical d. c. displacements as well as for the activation of the extrapyramidal system. According to the results of previous experiments the brain stem reticular formation possibly plays an essential rôle in mediating both these effects (cf. p. 31). In the majority of experiments the SP actions which were described above last about 1 hour. Both the duration and the intensity of the d. c. and EEG changes are, however, subject to some variation which is not due to differences in the applied dose.

In accordance with the experiment illustrated in Fig. 3 an i. p. application of 8,000 U./kg nearly always leads to a rapid and temporarily complete depression of the various evoked d. c. shifts. In order to study the process of extinction in greater detail fractionated SP injections up to the same final amount were therefore employed. According to these experiments the first visible effect usually obtained with 1,000—2,000 U./kg i. p. consists in a moderate slowing of the evoked potential rise. Increasing doses of SP additionally reduce the extent, the voltage and the duration of the d. c. deflections. Finally, the small persistent shifts of the cortical steady potential use to be restricted, more or less, to the primary projection field pertaining to the stimulus applied. Fig. 4 demonstrates a partial reduction of a peripherally induced negative d. c. displacement after i. p. application of 4,000 U./kg.

The employment of fractionated SP-injections furthermore reveals remarkable differences concerning the susceptibility of the negative d. c. deflections elicited by various peripheral stimuli. If the reduction of each negative shift is measured in per cent of its mean normal value an acoustically evoked d. c. reaction, for instance, proves by far more susceptible to SP than the negative deflections induced by tactile stimu-

lations of the animal. Among the various d. c. deviations elicited by natural stimuli conditioned reactions of the steady potential are particularly resistent to SP. This statement is illustrated in Fig. 5. In these records the proper stimulation response of the d. c. component (R) is preceded by a definite negative shift elicited by approaching the animal (A). In this special experiment the initial d. c. deviation represents the bioelectrical sign of a conditioned flight response established by the method of punishment. According to the tracings summarized in Fig. 5 the primary alarming reaction of the d. c. component (A) is clearly less reduced by SP than the unconditioned stimulation response (R). The concomitant activation of extrapyramidal motor structures, however, temporarily disappears even in this case (Fig. 5 B—C). As compared with the conditioned flight effects the spontaneous »approaching« reactions of the steady potential which frequently occur

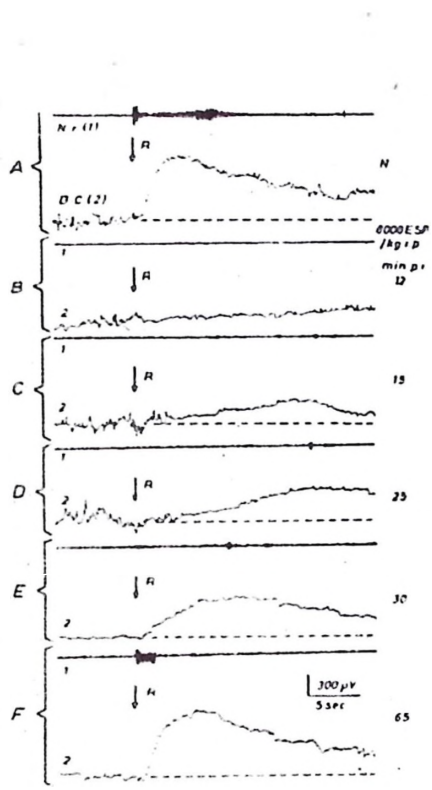


FIG. 3

The influence of SP on the neuronal activity of the nucleus ruber (N. r.; 1) and on the negative shift of the cortical steady potential (D. C.; 2) evoked by a tactile stimulation of the vibrissae (R). A: Normal stimulation responses. B-F: Extinction and recovery of the natural stimulation reactions after an i. p. application of 8,000 U./kg body weight.

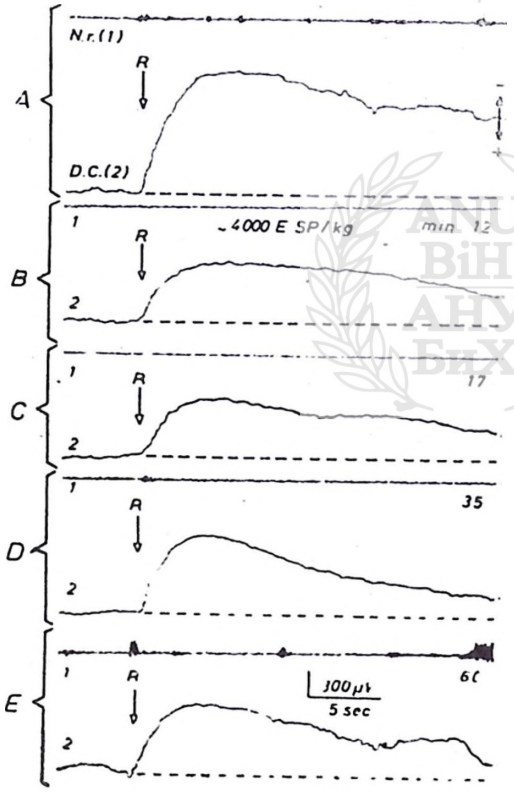


FIG. 4

The action of SP on the neuronal activity of the nucleus ruber (N. r.; 1) and on the negative shifts of the cortical steady potential (D. C.; 2) evoked by a tactile stimulation of the vibrissae (R). A: Normal stimulation responses. B-E: Reduction and recovery of the normal stimulation reactions after an i. p. application of 4,000 U./kg body weight.

also in normal animals without a special training (see Fig. 1) are somewhat less resistent to SP. Their reduction runs approximately parallel to the depression of the negative shifts evoked by tactile stimulations of the animal. As a whole, the alterations of various evoked d. c. shifts elicited by SP correspond in several aspects to the modification of conditioned and unconditioned behavioural phenomena which has been reported by Stern (1959).

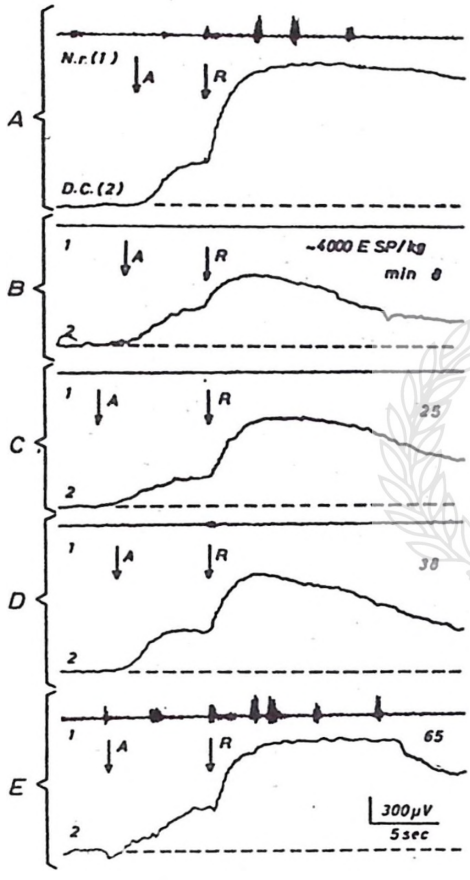


FIG. 5

The influence of SP on the neuronal activity of the nucleus ruber (N. r.; 1) and on the negative shifts of the cortical steady potential (D. C.; 2) evoked by approaching the animal (A) and by a subsequent stimulation of the whiskers (R). A: Normal stimulation responses. B-E: Reduction and recovery of the stimulation reactions after an i. p. application of 4,000 U./kg body weight. (for further explanations see text!)

III. **Some actions of SP on the behaviour of the rat.** — The changes of the cortical steady potential released by different doses of SP are closely related to typical behavioural phenomena which have already been observed in other animal species [v. Euler and Pernow (1956), Zetler (1956, 1959), Stern and Dobrić (1957), Stern and Huković (1958), Stern (1959) et al.]. The special features of the sedative effect depend on the applied dose. Smaller amounts of SP (i. e. 1,000—2,000 U./kg i. p.) usually cause a distinct reduction of spontaneity which can be distinguished from a physiological period of inactivity by a preceding analyses of the normal activity cycle of the

individual. In contrast to the spontaneous movements the motor responses of the animal to peripheral stimulations are, at first, scarcely altered. This statement especially proves true if »meaningful« stimuli are employed (cf. p. 35). Higher doses of SP (i. e. about 4,000 U./kg) usually effect a complete depression of any spontaneous motor activity, the reaction of the animal to peripheral stimuli concurrently being reduced. Such (medium) doses of SP occasionally provoke certain automatisms consisting, for instance, in chewing movements or in periodical risings of the head. Large amounts of SP exceeding 8,000—10,000 U./kg body weight finally cause a stuporous state with a temporary extinction of all spontaneous and induced motor actions. These findings are in general agreement with the observations made by von Euler and Pernow (1956) in cats and rabbits. In the majority of experiments the strong sedative effects produced by high doses of SP last about 30—45 min. and then vanish within the course of the subsequent hour. During this period the normal fluctuations of the cortical steady potential gradually reappear.

IV. **The direct actions of SP on the cerebral cortex.** — According to the results of previous investigations the extensive shifts of the cortical steady potential occurring with spontaneous or induced activity changes of the animal are mediated preferentially by the brain stem reticular formation (cf. p. 31). Therefore, the reduction of the evoked negative d. c. deflections could be due, in principle, both to a direct influence of SP on the cortical generator structures and to an inhibition of reticular neurons. A further analysis of the fundamental processes involved in the depression of the d. c. shifts can be achieved by comparing the activity changes of the cortex produced by a local and by an i. p. administration of SP.

As already observed in preceding experiments direct applications of SP to the cortical surface, excluding secondary actions of the substance via subcortical and extracerebral structures, exert a strong influence on the bioelectrical activity of the cortex (cf. Caspers and Stern, 1961). Various amounts of SP ranging from 1 to about 10 units per 10 mm^2 cortical surface cause a monophasically positive shift of the steady potential. The positive d. c. deviation is accompanied by typical variations of the direct cortical response (DCR) to a single electrical stimulus (so-called dendritic potential). Employing currents of low intensity a local application of SP always effects a distinct reduction of the DCR. The same amount of the substance, on the other hand, causes a significant increase of the DCR if stronger, »supra-threshold« stimuli are applied. Opposite SP effects of exactly the same kind depending on the current intensity are expressed in the negative d. c. displacements induced by a direct activation of the cortical surface with series of electrical pulses. In contrast to these d. c. deviations the natural shifts released by physiological stimuli are constantly

depressed at the site of application. On that account the neuronal afferent impulses impinging upon the cerebral cortex act like a weak direct electrical stimulation. In connection with the results of additional polarization experiments these findings, moreover, suggested that SP establishes a hyperpolarization of the structural elements which are responsible for generating both the d. c. component and the DCR.

Compared with the local actions of SP the various activity changes of the cortex occurring after an i. p. injection of the substance show some peculiarities. While a direct application of SP to the cortical surface, in either case, produces a monophasically positive deviation of the d. c. component intraperitoneal injections of the substance often release a secondary negative shift which sometimes even exceeds the initial positive displacement. An example of such an experiment was already illustrated in Fig. 2. Likewise, the changes of a supra-threshold direct cortical response following an i. p. application of SP may be entirely different. As a rule, medium amounts of the polypeptide up to 4,000 U./kg i. p. cause a distinct increase of the DCR (Fig. 6). This effect is identical with the local actions of the substance. Higher doses of SP, on the other hand, often reduce or abolish the DCR even if maximum stimuli are employed. An example of such a record is demonstrated in Fig. 7. The extinction of the (supra-threshold) DCR which was never observed after a local application of SP is closely related to the secondary negative shifts of the d. c. component and promptly abolished by an anodal polarization of the cortical surface. The negative deviations of the steady potential, on their side, always appear in connection with a lowering of blood pressure elicited by a sufficient amount of SP. They rather constantly start whenever the mean systemic pressure declines to a certain minimum value which varies to some extent from one individual to another. Negative d. c. shifts of exactly the same form which are accompanied by a reduction or a suppression of the DCR also occur when the critical fall in blood pressure is initiated by substances other than SP. Taking into account the results of earlier experiments, it may be assumed that the negative displacements of the steady potential depend upon an increasing depolarization of the cortical generator membranes released by hypoxia. On this condition the negative DCR which is induced by an additional electrical stimulus necessarily decreases or disappears independently of the stimulus strenght employed. The negative shift of the surface steady potential as well as the reduction of the DCR, therefore, obviously represent secondary effects of SP which are mediated by changes in circulation. Consistent with the results of previous experiments (cf. Caspers and Stern, 1961) both the positive d. c. deviations and the increase of the DCR, on the other hand, can be attributed preferentially to a direct action of SP on the cerebral cortex.

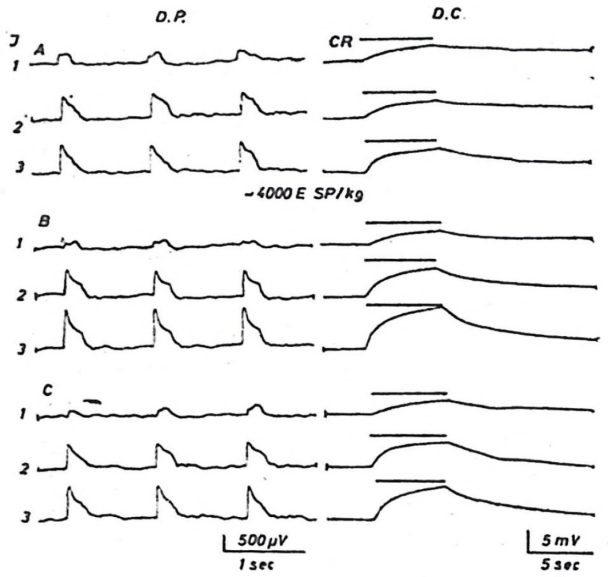


FIG. 6

The action of SP on the direct cortical response (so-called dendritic potential; D. P.) and on the negative shift of the steady potential (D. C.) induced by an electrical stimulation of the cortical surface with a 20/sec. impulse series. The time of stimulation is indicated by horizontal lines (CR) above each d. c. record.

A: Normal D. P. and D. C. responses to electrical stimuli of increasing intensity (I 1; I 2; I 3). B-C: Variations and recovery of the normal stimulation responses of the cerebral cortex 10 min. (B) and 50 min. (C) after an i. p. injection of 4,000 U./kg body weight. (for further explanations see text!)

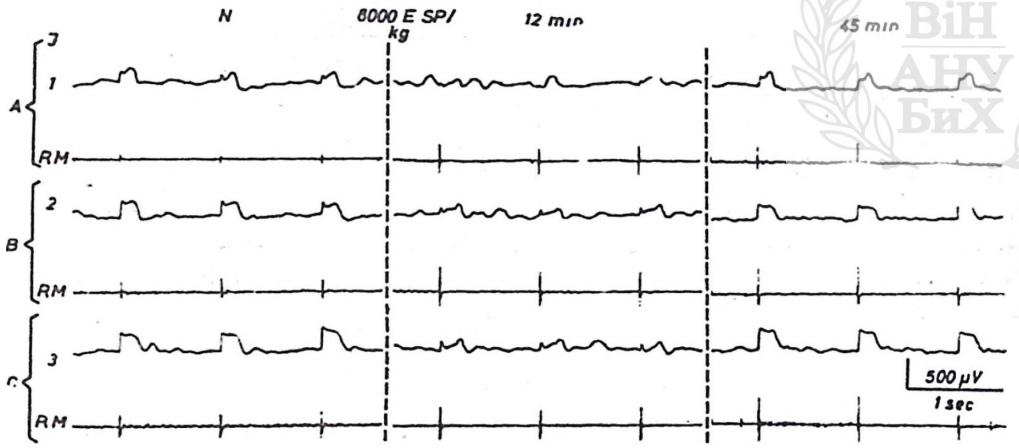


FIG. 7

The action of larger amounts of SP on the direct cortical response to a single electrical stimulus. The moment of each stimulation is indicated by the vertical lines in RM. N: Normal response of the cerebral cortex to stimuli of increasing intensity (A, B, C). The medium column of the figure demonstrates a considerable reduction of the normal response at all current intensities 12 min. after the i. p. application of 8,000 U./kg body weight. The right column of tracings shows a recovery of the effect 45 min. after the injection. (for further explanations see text!)

According to the results discussed above any reliable conclusion concerning the main site of the SP-actions can be derived only from those experiments in which a substantial lowering of blood pressure is missing or in which it is possible to compensate the effects on cir-

culation by means of medicaments which do not influence, at least to a measurable extent, the functions of the brain themselves. These special conditions proved accomplished, more or less, in a total of seven tests. As could be calculated from the various records injections of SP just sufficient to abolish each evoked negative d. c. shift release a positive deviation of the d. c. base line up to a maximum of 4 mV. The concurrent increase of the direct cortical response to a suprathreshold stimulus amounts to 30 per cent of the initial value. A local administration of SP, on the other hand, which also suppresses all of the evoked negative d. c. deflections at the site of application causes a positive displacement of the d. c. base line up to 8 mV and increases the DCR up to 80 per cent of its mean normal voltage. These comparative measurements indicate that the reduction or extinction of the peripherally evoked d. c. deviations following an i. p. injection of SP cannot be completely explained by a mere cortical action of the substance. A significant part of these effects is apparently due to an inhibition of reticular activity.

V. Some actions of SP on the activity of the brain stem reticular system. — The assumption that SP is apt to inhibit reticular neurons was examined, at first, in some additional stimulation experiments. As already observed by various authors [cf. Arduini et al. (1957), Goldring and O'Leary (1957), Brookhart et al. (1958), Caspers (1959)] high frequency stimulations of the brain stem reticular system elicit quite similar negative shifts of the cortical steady potential as are released by natural stimuli. According to the results of more recent investigations two different types of the cortical d. c. response to an electrical activation of reticular structures are to be distinguished (Caspers and Schulze, in press). Besides steep deviations of the steady potential which arise from the base line immediately at the commencement of the reticular stimulation, flat shifts of considerable latency occur. An example of the slow type of stimulation response is illustrated in Fig. 8. This form of reaction is usually obtained with the electrodes located in the lateral portion of the mesencephalic reticular system, while the first, short latency type predominates in the median part. Both forms of the electrically evoked d. c. displacements prove rather susceptible to SP, the slow type being the most sensitive one. As a rule, it disappears after an i. p. application of 4,000—8,000 U./kg (Fig. 8). In either case, however, the negative shifts of the cortical steady potential induced by a reticular activation are completely depressed when a direct stimulation of a thalamic relais nucleus, for instance, still effects distinct d. c. alterations. These findings suggest that the reticular system is particularly sensitive to SP, and that a lowering of the reticular excitation level may actually be involved in the production of the sedative effects which are reflected in the d. c. alterations at the cerebral cortex. The records summarized in Fig. 9 yield a direct evidence of this interpretation. The subsequent tracings demonstrate that both the basic activity level and the stimu-

lation response of reticular neurons are clearly depressed by SP. The normal responsiveness of the recorded units is gradually restored within the course of 1 hour following the application of SP. In this experiment the recovery curve of the reticular activation runs parallel to the recreation of the peripherally evoked d. c. shifts. As a whole,

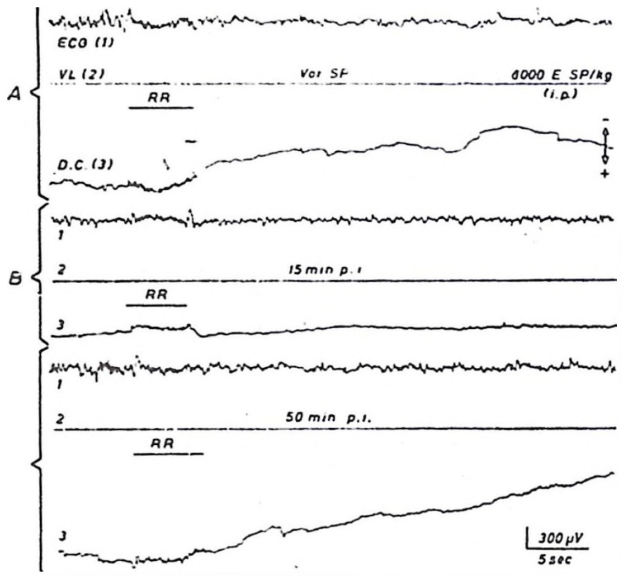


FIG. 8

The action of SP on the electrocorticogram (ECG) and on the negative shift of the cortical steady potential (D. C.; 3) released by a direct stimulation of the lateral portion of the midbrain reticular formation. VL (2) presents a comparison line. The period of reticular stimulation in each tracing is indicated by the horizontal lines RR. A: Normal stimulation response. B-C: Extinction and recovery of the evoked (long-latency) deviation of the cortical d. c. component after the i. p. application of 8,000 U./kg body weight SP.

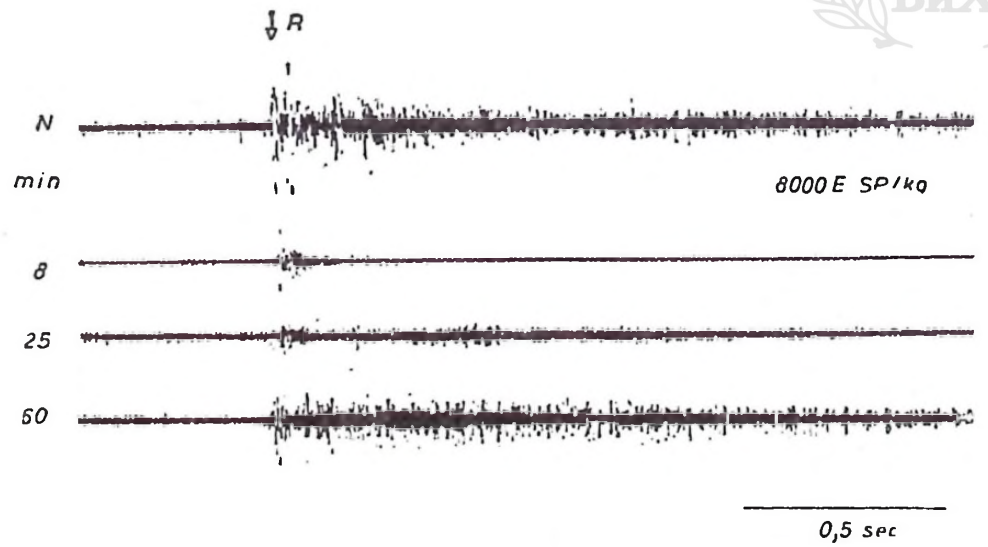


FIG. 9

The action of SP on the unit discharges of neurons in the midbrain reticular formation evoked by a tactile stimulation of the vibrissae. The moment of stimulation in each tracing is indicated by the arrow R. Both the level of the background activity and the normal stimulation response (N) of reticular neurons recorded with a 50 µ electrode are strongly reduced after the i. p. application of 8,000 U./kg. The normal response has recovered approximately after 60 min.

the investigations allow to conclude that the depression of cortical excitation processes following an i. p. injection of SP can be explained only in part by a direct action of the polypeptide on the cortical generator structures. A significant component of the sedative effects reflected in the extinction of the evoked cortical d. c. shifts consists of an inhibitory action of SP on reticular neurons.

Summing up, the experiments have shown that the depression of spontaneity released by a parenteral application of SP is actually accompanied by typical activity changes in the reticulo-cortical system. As could be concluded from the various d. c. alterations both the basic activity level and the responsiveness of the cerebral cortex to different natural stimuli are greatly reduced. With respect to the stimulation reaction of the reticulo-cortical system, SP acts, as a whole, in a way as if the amount of »information« implied in a given peripheral stimulus had been diminished. The fundamental neurophysiological mechanisms responsible for these SP effects are, as yet, unknown. Some experimental data support the hypothesis proposed, for instance, by Zetler (1959) that the polypeptide represents a transmitter substance at certain inhibitory synapses. The conclusion of Caspers and Stern (1961) that SP causes a hyperpolarization of neuronal structures would favour this interpretation. A direct evidence of the assumption that the polypeptide might be involved in the transmission of inhibitory impulses is, however, entirely lacking. At the present stage of our knowledge we would prefer to assume that SP represents a hyperpolarizing, permanently active agent, the neuronal actions of which being more diffusely distributed.

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Summary

The question of how SP is influencing the stimulation processes in the cortex and in the reticular formation of the brain stem has been investigated on freely moving rats with chronically implanted electrodes. The direct potential component (Bestand-potential) of cortical bioelectric activity, the so called dendritic potential, the spontaneous waves of the EEG and the neuronal discharges in the mesencephalon reticular structures served as criteria for SP action. Bioelectric effects and the known sedative action of SP have been correlated simultaneously. The experiments have shown:

(1) After i. p. administration of about 2,000—4,000 U./kg SP the cortical direct potential is shifted to the positive side of the initial value for several mV. The assuming of more positive potentials by the cortex is connected, similarly as in natural sleep, with an increase of the so called dendritic potential and the spontaneous waves of the EEG. After administration of h i g h e r doses of SP the primary positive shift may quickly change over into a negative one. Such negative potential shifts in the cortex which are lacking when SP is applied locally indicate a close relationship to the blood pressure reduction

produced by higher doses of SP. They obviously represent secondary effects in the cortex, depending on extracerebral action of SP.

(2) The shift of Bestandpotential appearing after an injection of SP is always coupled with a reduction of fluctuations of negative direct potential which, in awake, freely moving animals, results from sensory stimulation. The depression of response to stimuli, measured in per cent of the initial value, increases with increasing doses of SP. The depression, further, depends on the kind of stimulus applied. With equal dosage of SP the per cent reduction of direct potential fluctuations is generally the smaller, the more »information« for the individual under examination there is contained in the sensory stimulus.

(3) The inhibition of cortical stimulation processes as reflected in direct potential tracings is partly due to direct action of SP in the cortex. But according to comparative measurements carried out with intraperitoneal and local administrations of SP this inhibition cannot entirely be explained by its direct action. Another essential cause of cortical inhibition effects rather lies in a lowering of the reticular stimulation level. As shown by direct recording of neuronal discharges in the brain stem reticular formation the spontaneous activity, as well as the response to stimulation of reticular structures are considerably reduced by SP.

From the results obtained it might be concluded that the suppression of stimulation processes in the reticulo-cortical system takes a significant part in the appearance of the well-known sedative effects of SP.

NEKI EFEKTI SP U MOŽDANOJ KORI I RETIKULARNOJ FORMACIJI MOŽDANOG DEBLA

Na slobodno pokretljivom štakoru kome su kronično implantirane elektrode istražen je način djelovanja SP na stimulativne procese u moždanoj kori i retikularnoj formaciji moždanog debla. Kao kriteriji za prosudivanje djelovanja SP poslužili su: istosmjerna komponenta (Bestandpotential) bioelektričke aktivnosti moždane kore, zatim tzv. dendritni potencijal, spontani talasi EEG i neuronska izbijanja u retikularnoj formaciji. Istovremeno su ove bioelektričke manifestacije povezane s poznatim sedativnim djelovanjem SP na ponašanje životinja. Eksperimenti su pokazali:

(1) Poslije intraperitonealne aplikacije oko 2.000 do 4.000 jed./kg SP, istosmjerni kortikalni potencijal pomiče se za nekoliko mV na pozitivnu stranu ishodne vrijednosti. Pojava pozitivnog potencijala u moždanoj kori ide u korak s povećanjem tzv. dendritnog potencijala i spontanih talas EEG slično kao kod prirodnog spavanja. Poslije v e ć i h doza SP može prvobitni pozitivni pomak kortikalnog potencijala ubrzo preći u negativni. Nastup takvih negativnih potencijala moždane kore, kojih nema pri lokalnoj aplikaciji SP, usko je povezan sa smanjivanjem krvnog pritiska što ga izazivaju veće doze SP. To su, dakle, očito sekundarni efekti u moždanoj kori, uvjetovani ekstracerebralnim djelovanjem SP.

(2) Pomaci istosmjernog kortikalnog potencijala, koji nastupaju poslije injekcija SP, uvijek su skopčani sa smanjivanjem fluktuacija negativnog istosmjernog potencijala, koje su kod budne, slobodno pokretljive životinje posljedica senzornih podražaja. Smanjivanje reakcije na podražaj, izraženo u procentima početne vrijednosti, raste s porastom doze SP. Pored toga, ono

ovisi i o vrsti podražaja. Uz jednake doze SP procentualno sniženje istosmjernih fluktuacija općenito je utoliko manje ukoliko je veći sadržaj »informacija« u primijenjenom senzornom podražaju.

(3) Kočenje kortikalnih stimulativnih procesa, koji dolaze do izraza u registrovanim crtežima istosmjernog potencijala, djelomično je zasnovano na direktnom djelovanju SP u moždanoj kori. Medutim, prema rezultatima uporednih mjerenja pri intraperitonealnoj i lokalnoj aplikaciji SP nije mogućno protumačiti ovo kočenje isključivo direktnim djelovanjem SP. Štaviše, sniženje retikularnog podražajnog nivoa predstavlja dalji bitan uzrok kočenja kortikalnih podražajnih procesa. Kako to pokazuje direktno registrovanje neuronskih izbijanja u retikularnoj formaciji moždanog debla, djelovanje SP znatno smanjuje i spontanu aktivnost i reakcije na podražaj u retikularnim strukturama.

Rezultati ovih istraživanja dopuštaju da se zaključi da kočenje stimulativnih procesa u retikulo-kortikalnom sistemu bitno sudjeluje u tvorbi poznatog sedativnog efekta SP.

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DISCUSSION

KRIVOY: Where were the electrodes located?

CASPERS: The d. c. potentials were led from various areas of the intact cortical surface and recorded against a reference point in the front portion of the muzzle which has proved sufficiently inactive.

v. EULER: Pernow and I have noted the effects of SP lasting for more than 1/2 hour after administration in the brain ventricles, indicating a slow inactivation.

CASPERS: Our observations on rats are in good agreement with your experimental results. We found that the direct cerebral effects produced by an i. p. injection of a medium dose of SP (cf. p. 37.) usually last as long as 30—60 min. after the administration. This finding, too, points to a slow inactivation of the substance.

STURMER: In terms of our highly purified SP, a dose 4,000 U./kg rat by injection corresponds to 120 µg/kg which cannot be regarded as a large dose.

Polypeptides like oxytocin, vasopressin or bradykinin are destroyed in a matter of minutes when injected. It is remarkable therefore that the effect of parenteral SP should last for about 0.5 hour.

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B. PERNOW

SOME EFFECTS OF SUBSTANCE P IN MAN

In the original report by Euler and Gaddum (1931) SP was defined as a smooth muscle stimulating and blood pressure depressor factor, and we know now that these two effects are also obtained with the more purified preparations, the one containing 3,000 units per mg (Pernow, 1953) and now the chemically pure one, containing 30,000 units per mg (Franz, Boissonnas and Stürmer, 1961). Both effects are also observed in man following intravenous administration of SP and the present paper deals with some observations on the actions of SP on circulation and the intestinal muscles.

I. EFFECTS ON THE CIRCULATION

The typical response following intravenous administration of SP is a rapid fall in blood pressure. This effect, which is not blocked by either atropine, antihistaminics or ganglionic blocking agents, is obtained in all mammals and is due mainly to a peripheral vasodilatation (Euler and Gaddum, 1931; Euler, 1936). In the isolated rabbit ear Holton and Holton (1952) observed a vasodilatation following intraarterial administration of SP.

In the first study on the effect of SP in man (Liljedahl, Mattsson and Pernow, 1958) an immediate rise in pulse rate and fall in arterial blood pressure was observed following intravenous infusion of SP. A bright red flush in the face was noticed as a characteristic effect. All subjects felt throbbing sensations in their head. All reactions were transient and disappeared even during continued infusion. It was then possible to increase the infusion rate three to four times without any further reactions.

In order to study this preliminary observation more in detail the effect of SP on the central hemodynamics, splanchnic and forearm blood flow was studied separately. The SP preparation used had an activity of 550 Euler units per mg. It was dissolved in saline and given intravenously in amounts of 0.2—1.2 U./kg/min.

A. General symptoms

Immediately after starting the infusion the subjects felt warmth in their head and temporal pulsations. Simultaneously a bright red flushing was observed in the face, neck and arms. These symptoms

reached a maximum 1—2 min. after the start of infusion, lasted another 2 min. and then slowly disappeared in spite of a constant infusion rate. In some cases the symptoms returned, but usually to a milder degree, when the infusion rate was increased. In others the infusion rate could, after a few min., be increased manyfolds without any further symptoms. No further reactions were observed.

B. Effect on central circulation

(Dunér and Pernow, 1959)

Methods

These effects were studied in connection to heart catheterizations in healthy male volunteers. A double lumen Cournand catheter was inserted into the right heart from a cubital vein. A polyethylene catheter was put percutaneously into a brachial artery. Pressure recordings were obtained from the right ventricle, pulmonary artery, pulmonary arterial wedged position (PCV) and the brachial artery. Cardiac output was determined by the direct Fick's method, that is on ground of the oxygen uptake in the lungs and the difference in oxygen between arterial and mixed venous blood. Expired air was collected in Douglas bags. Blood oxygen content and capacity were analyzed spectrophotometrically.

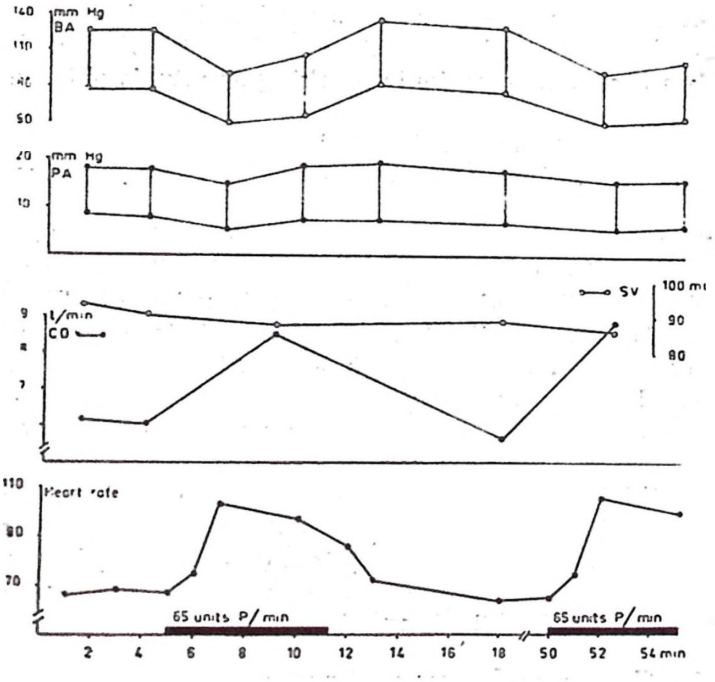


FIG. 1

Effect of repeated intravenous infusions of substance P on the central hemodynamics in man. BA = brachial artery, PA = pulmonary artery, CO = cardiac output, SV = stroke volume (Dunér and Pernow).

Results

Heart rate. — The heart rate immediately increased during infusion of SP in doses of 0.3 U./kg/min. or higher. The tachycardia was maxi-

mal 2 min. after the start of infusion. Normal pulse rate was resumed 1—2 min. after stopping the infusion.

Blood pressure. — Right ventricular, pulmonary artery and PCV pressures did not change significantly during SP infusion. The brachial artery pressure, however, dropped immediately after the start of infusion. Minimal values were recorded within 2 min., after which the arterial pressure again increased even during continued infusion. When the infusion was repeated 15—30 min. later, the same fall in arterial pressure, as well as in heart rate was, however, observed.

Oxygen uptake. — No change was observed in pulmonary oxygen uptake during SP infusion.

AV-O_2 difference. — During SP infusion the total arterio-venous oxygen difference, as calculated from brachial and pulmonary artery blood, decreased slightly.

Cardiac output and stroke volume. — At an infusion rate of 0.4 U./kg/min. and more the cardiac output increased slightly in all cases. This increase was entirely due to the increased heart rate since the stroke volume was usually formed unchanged during the infusion.

C. Effects on splanchnic blood flow

(in collaboration with Castenfors, Eliasch and Hultman)

Method

The method used was the dye clearance technique introduced by Bradley. Bromsulphalein was used as a dye, which is known to be exclusively eliminated by the liver. The dye was given in a constant i. v. infusion. It is known that the rate at which the liver can remove the dye is proportional to the concentration. When the rate of removal becomes equal to the rate of infusion, the blood level stabilizes, and, therefore, the rate of removal can be determined indirectly by measuring the rate of infusion. If the rate of removal and the proportion of dye removed by the liver from each milliliter of blood is known, the blood flow is simply determined by the relation

$$\text{blood flow} = \frac{\text{amount removed per min.}}{\text{amount removed from each ml of blood.}}$$

The technique therefore necessitates catheters in an artery and a hepatic vein. The assumptions of this clearance technique are that the liver solely removes the dye, and that the concentration of the dye obtained by catheter from the hepatic vein is representative of all of the blood leaving the liver.

Results

I. v. infusion of SP gave rise to a slight increase in splanchnic blood flow which was raised from 1,540 to 1,830 ml per min. Simultaneously the concentration of bromsulphalein increased slightly in both

arterial and hepatic venous blood at unchanged infusion rate, indicating a decrease in removal of dye by the liver. The explanation of this is not known but might suggest a shunting of dye through arterio-venous anastomoses in the liver.

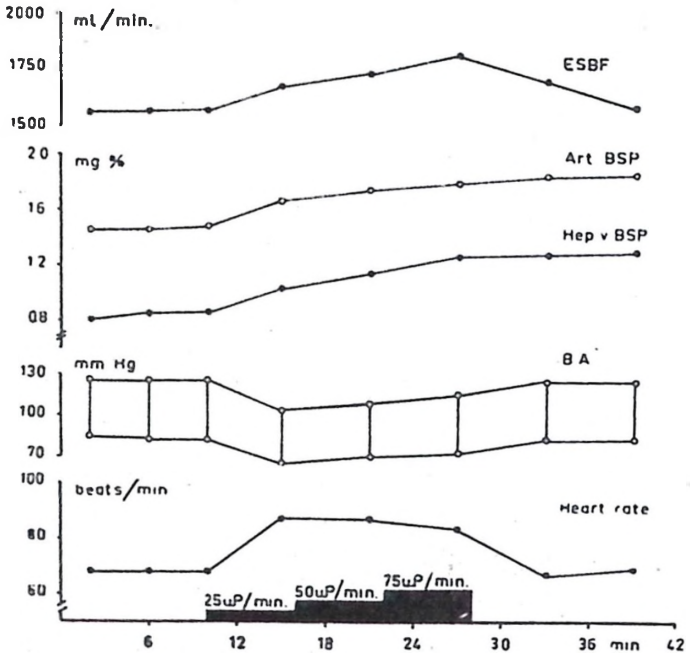


FIG. 2

Effect of intravenous infusion of substance P on the heart rate, brachial artery pressure (BA), bromsulphalein concentration of the brachial artery (Art. BSP) and hepatic vein (Hep. v. BSP) and the estimated splanchnic blood flow (ESBF) (Castenfors, Eliasch, Hultman and Pernow)

D. Effect on the blood flow of the forearm

(in collaboration with Wahren)

Method

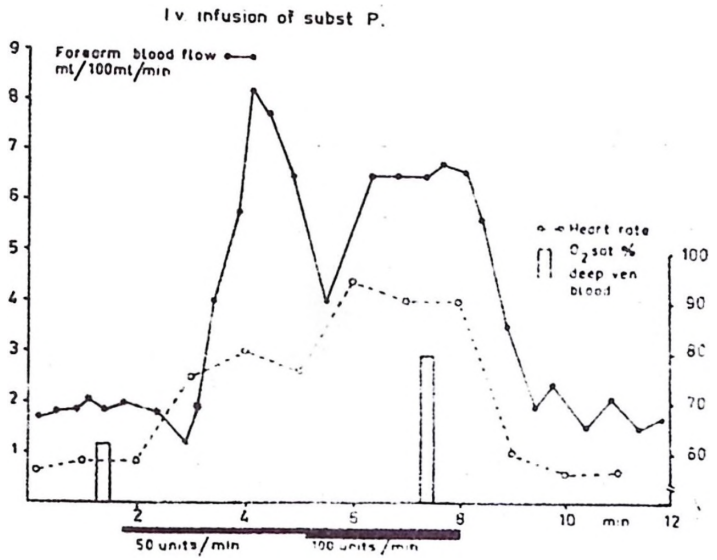
The observations were made on healthy male subjects lying in supine position. Forearm blood flow was measured by venous occlusion plethysmography as described by Graf and Westergren (1959). A polyethylene catheter was inserted percutaneously in a cubital vein and put about 8 cm in the distal direction into a deep vein, which is known to give practically pure muscle blood.

Result

After a latency of about 2 min. the usual increase was obtained in heart rate. The typical bright red flush was observed in the face, neck and arms. The forearm blood flow increased rapidly to maximum five times the resting level. A significant rise in oxygen saturation of the deep venous blood was obtained, indicating an increase in muscular blood flow, since the oxygen uptake of the muscle was supposed to be unchanged (Fig. 3).

FIG. 3

Effect of intravenous infusion of substance P on the forearm blood flow, the oxygen saturation of deep venous blood of the forearm and the heart rate (Pernow and Wahren).



Conclusions

Intravenous infusions of partly purified SP preparations were shown to elicit significant effects on the circulation in man. The predominant effect seems to be a peripheral vasodilatation with a fall in arterial blood pressure. An increased blood flow was observed specially in the muscles and skin, while the splanchnic blood flow was only moderately raised. The dilatation of the skin vessels caused a flushing which was best observed in the head and arms. Cardiac output was increased due to a raised heart rate, whereas the stroke volume was unchanged. The tachycardia was probably due to the rapid fall in blood pressure.

II. THE EFFECT ON THE INTESTINAL MOTILITY (Liljedahl, Mattsson and Pernow, 1958).

The most striking effect of SP is its stimulating effect on smooth muscle, specially that of the intestinal wall. This has been shown both on isolated segments and intestine »in situ«.

1. Studies on isolated segments of human intestine. Pieces of gut were obtained at operations and were, immediately after removal, placed in Tyrode's solution and, later on, suspended in an ordinary intestinal bath. Both duodenal, jejunal, and ileal segments were found to be sensitive to SP, the duodenum however about 4—5 times less sensitive than the other segments mentioned. In the ileum preparations 0.3 units per ml bath fluid produced a contraction about twice the spontaneous amplitude.

The action of SP consisted in an initial relaxation, rapidly followed by a gradual increase in tone. Small concentrations of SP usually pro-

duced an increase in the spontaneous movements only, while larger amounts gave rise to an increased tone. The slow-reacting type of contraction, typical for SP on all smooth muscle organs, is quite distinguishable from the rapid response produced for instance by ACh.

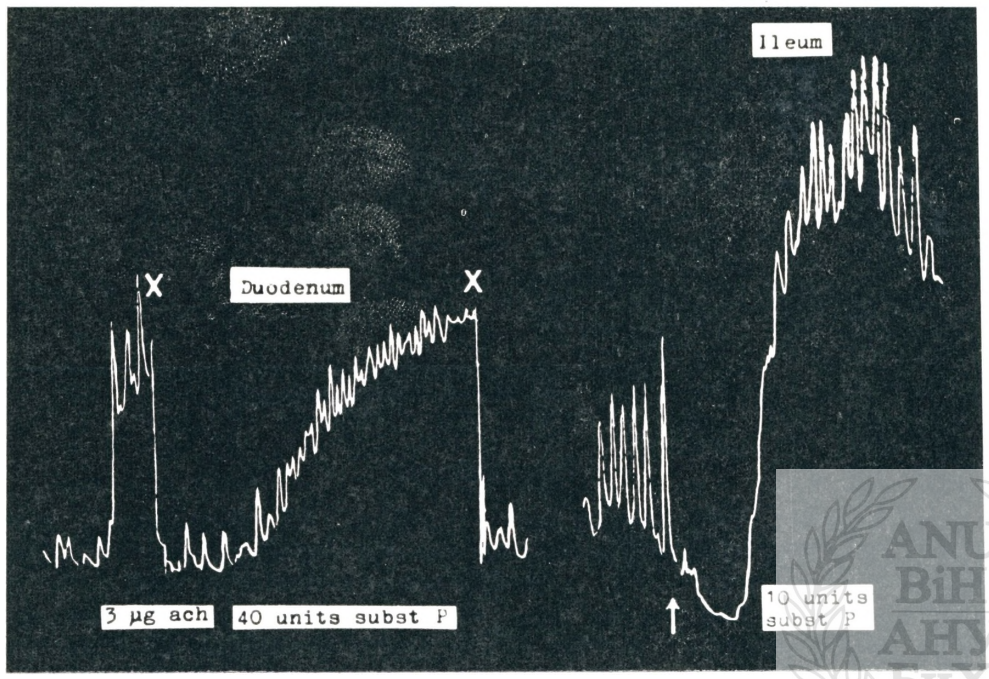


FIG. 4

Effect of substance P and acetylcholine on the isolated human, duodenum and ileum. Bath volume, 15 ml. At X, changing of the bath fluid (Liljedahl, Mattsson and Pernow).

2. Effect of intravenous infusion of SP on intestine »in situ« (illustrated by a film). The radiographic recordings were performed with a cinecamera, type Arriflex (35 mm film) connected to a Philips image amplifier. The camera speed was constantly 16 frames per second. To obtain constant exposure of the film during the whole examination, independently of the varying object thickness, amount of contrast media etc., a millivoltmeter was connected to the intensifier. By this device the amount of light emanating from the intensifier could be evaluated directly and the X-ray intensity be adjusted accordingly. The recordings were, as a rule, made in consecutive periods of 10—15 seconds. Screen examinations were made in the meantime in short periods and at low X-ray intensity.

Intravenous infusions of 1—1.5 mg of SP (600—1,000 units) during 20-min. periods elicited within 5 min. an increase in intestinal motility. This change was demonstrable for both the segmental and the peristaltic movements. In the fluoroscopic picture local deep constrictions of the intestinal lumen predominated, which indicated powerful con-

tractions of the circular muscles. In some areas the intestinal lumen diminished markedly, which suggested a general rise in tone of the intestinal muscles. Now and then powerful, prolonged peristaltic waves were seen, which forced the barium content of the intestine in the anal direction. The increased motility was observable during the whole period of infusion, and for 10—20 min. after the end of infusion.

In a case of intestinal paralysis the gut was initially enormously distended and showed no types of movements. The patient then got 1,000 units SP during a 20-min. infusion. Eight minutes after the start of infusion a few segmental contractions rapidly changed the shape of the intestine, and were followed by adequate peristaltic waves extended over about 10—20 cm of the intestine in an aboral direction. This activity, however, became fainter, even during continuous infusion, and disappeared rapidly after the end of infusion.

Summary

Highly purified substance P preparations were infused intravenously in healthy human subjects. The dominating circulatory effect was a peripheral vasodilatation with increase in muscle and skin blood flow, flushing and fall of the arterial blood pressure. No effect was obtained on the blood pressures of the right heart. Heart rate and cardiac output increased.

The effect of substance P on the intestinal motility was studied both on isolated segments and intestine in situ. A marked increase in both segmental and peristaltic movements were observed, which was true both for normal subjects and in patients with intestinal paralysis.

NEKI EFEKTI SP KOD ČOVJEKA

Zdravim licima davao se vrlo prečišćeni preparat SP putem intravenske infuzije. Periferna vazodilatacija s porastom protoka krvi kroz mišiće i kožu, pojavom crvenila i padom arterijskog krvnog pritiska, nastupila je kao dominantni cirkulatorni efekat. Nikakvo djelovanje nije zapaženo na krvni pritisak desne strane srca. Srčani ritam i srčani volumen bili su povećani.

Djelovanje SP na pokretljivost crijeva studirano je na izolovanim crijevnim segmentima, kao i na crijevu in situ. Zapaženo je znatno pojačanje segmentskih i peristaltičkih pokreta, i to kako kod normalnih lica tako i kod pacijenata s intestinalnom paralizom.

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DISCUSSION

HUKOVIĆ: Do the symptoms decrease during continued infusions of SP? What is it the subject feels like during the infusion of SP? The injection of crude SP in mice is followed in our experiments by a weakness of the skeletal muscles and, with great doses of SP, the complete paralysis of skeletal muscles could be seen *in vivo*. When you injected SP to yourself, could you feel any muscular weakness? Do you think that the changes in the blood flow could be due to the changes of muscle contractility?

PERNOW: Dr. Huković asked about the decrease in symptoms and effects during continued infusion. The effect was not due to a decrease in activity in the infusion solution, which was checked by assaying a proof of the infusion fluid after the end of the experiment. The decrease in effect seems to be rather due to a tachyphylaxis and is not specific for SP. If I am right, the same thing is seen for instance with H: intravenous infusion of H in the cat gives rise to an immediate fall in blood pressure, which is successively returning to the initial level, even during continued infusion. We never observed any muscular weakness during SP administration. I do not think that the effect of SP on the blood flow could be due to an effect on the muscular contractility since these studies were performed on resting subjects and since the blood flow increased not only in the muscles but also in the skin.

EULER: It would be of interest to study the content of SP in the intestine after periods of vagal stimulation. Preliminary experiments on rabbit have suggested that the vagus stimulation is followed by increased amounts of SP in the gut.

АНУ ВіН АНУ БиХ

B. PERNOW AND U. S. v. EULER

EFFECT OF INTRAVENTRICULAR ADMINISTRATION OF SUBSTANCE P IN THE UNANAESTHETIZED CAT (FILM)

Technique. — A cannula was implanted in the lateral ventricle through a parasagittal incision in the parietal bone according to Feldberg and Sherwood (1954). The location of the needle tip was checked by the passage of cerebrospinal fluid through the needle and by injection of a dye, the distribution of which was studied post mortem. The operation was performed one month before the experiments shown on the film.

Preparation. — The SP preparation used was prepared from cow's intestine and purified according to Pernow (1953). The activity was 300 units per mg. For control purposes inactivated preparations were obtained by incubation with chymotrypsin.

Results. — After administration of 30 units SP in 0.1 cc Ringer the dominating effect was a general inhibition of spontaneity. The cat lay down in a squatting position with the eyes half shut and showed almost no reactions when interfered with. This effect lasted about 1 hour, whereafter the normal behaviour of the cat was successively normalized. Besides these symptoms of general sedation the injection caused hyperpnea, licking and swallowing movements.

The experiment was repeated with 50 units SP in 0.15 cc Ringer. Again the immediate and dominating reaction was a general inhibition of spontaneous activity. About 30 min. the cat remained quite immobile in a state of stupor and was not affected by changes in its position. Except for occasional vigorous licking movements no other symptoms were noticed. At this occasion the stupor was followed by a short period of aggressivity with bad temper before the cat returned to her normal state.

No effects were observed after injection of Ringer's solution or inactivated SP.

Conclusion. — The most regular effect, which was elicited by intraventricularly administered SP was a general sedation. Similar effects were at the same time as this film was taken observed by Zetler (1956) after subcutaneous administration of SP in the rat. These observations have later been confirmed by Stern and Dobrić (1957).

Summary

Intraventricular administration of substance P caused an inhibition of spontaneous activity in the cat.

EFEKAT INTRAVENTRIKULARNE APLIKACIJE SP KOD MAČKE PRI PUNOJ SVIJESTI (FILM)

Intraventrikularna aplikacija SP prouzrokovala je kod mačke inhibiciju spontane aktivnosti.

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F. LEMBECK

LACK OF INTERACTION BETWEEN SUBSTANCE P AND DRUGS ON THE INTESTINE

The interaction between a pharmacologically active substance occurring in the tissue and a drug is usually reflected

(a) by a modification (potentiation, inhibition) of its action by the drug,

(b) by a change of its concentration in the tissue under the influence of a drug.

In the central nervous system an interaction between SP and several centrally acting drugs has been observed (Zetler, 1956, 1959, 1960). The amount of SP in the brain also depends to some extent on the interaction of various drugs (Zetler and Ohnesorge, 1957; Stern and Kocić-Mitrović, 1960). But so far no informations are available concerning interactions between SP and other compounds on the intestine. As Dr. Huković reports at this symposium, a large number of drugs has been tested for possible inhibitory effects on the action of SP. With the still doubtful exception of two substances no drug has been found, which specifically inhibits the action of SP. We tried bradykinin, oxytocin, and kallidin, but they were ineffective, too. The only substance which has been found to inhibit the action of SP was SP itself when given in large doses. This effect is due to tachyphylaxis.

Dr. Petschke and I investigated whether the activity of SP in the small intestine of the rat could be influenced by drugs. Morphine, physostigmine or atropine were injected to rats 30 min. later the animals were sacrified and extracts were made from the intestine. No differences in the SP content between the three groups were found. An influence of the intestinal motility on the SP content of the gut would possibly have been revealed by these experiments, since the gut of the physostigmine-treated animals was strongly contracted and empty, in contrast to the other two groups. The tissue content of a substance does, however, not allow conclusions about a change in rate of synthesis or release as long as synthesis and release of this substance go parallel.

The lack of specific inhibitors may lead to the assumption of a specific receptor for SP. It also could be that the mode of action of

ail the smooth muscle stimulating peptides differs from that of the smooth muscle stimulating amines since no inhibitors of the action of the other peptides are known, too. The experiments on the tissue concentration of SP suggest that factors other than the motility are more likely to influence the intestinal content of SP.

Summary

In contrast to the central actions of SP its effect on the smooth muscle could not be influenced by antagonists in a specific manner except by the tachyphylaxis observed after high doses of SP.

Whereas some drugs had effects on the SP concentration of the brain, drugs exerting an influence on the motility of the gut were found to be without an effect on the SP concentration of the intestine.

IZOSTAJANJE UZAJAMIČNOG DJELOVANJA SP I LIJEKOVA U ODNOSU NA CRIJEVO

Za razliku od centralnih efekata, efekti SP na glatke mišiće ne podliježu utjecaju specifičnih antagonista, izuzevši tahifilaksiju, koja se zapaža poslije velikih doza SP.

Dok neki lijekovi imaju utjecaja na koncentraciju SP u mozgu, lijekovi koji utječu na motilitet crijeva nemaju nikakvog utjecaja na koncentraciju SP u crijevu.

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B. PERNOW AND S. WALLENSTEN

THE RELATIONSHIP BETWEEN SUBSTANCE P AND THE MOTILITY OF THE SMALL INTESTINE IN MAN

A preliminary report

In the discussion about the physiological significance of SP in the intestinal tract it has been supposed that the substance is in some way related to the intestinal motility, in view of its special appearance in those parts of the gut having the highest degree of motility, and further its powerful stimulating effect on the intestinal smooth muscle. In 1952 Ehrenpreis and Pernow found at least some correlation between the degree of motility and SP concentration in a material of Hirschsprung's disease. Thus it was shown, that the proximal part of the recto-sigmoid, which is neurohistologically quite normal and hyperactive, had a significantly higher SP concentration than the corresponding segment in normal rectum. Furthermore, the distal aperistaltic and aganglionic part of recto-sigmoid in the Hirschsprung series showed much less SP than the control segments.

In the present study we have approached this problem by analyzing pieces of jejunum and ileum for their content in SP, before and after provoked increase in motility.

Procedure. — At abdominal surgery a duodenal catheter was introduced down to jejunum and 150 ml of 50 per cent glucose solution was applied locally. Prior to the glucose administration a small piece of jejunal tissue was taken out. The defect was sutured. The glucose solution was then administered and the resulting change in motility observed. When it reached its peak, usually 4—5 min. after injection, another piece of gut of equal size was excised adjacent to the first.

Extraction method. — The pieces of gut were, immediately after removal, put into acetone and extracted according to Amin, Crawford and Gaddum (1954). The SP activity was assayed on isolated guinea pig ileum in the presence of atropine, Lergigan (antihistamine) and tryptamine. The activity was completely inactivated by trypsin.

Material. — The investigation was carried out in 10 patients who should be operated upon with partial gastrectomy. Another series consisted of 8 patients with dumping syndrome of such a severity that they had to be reoperated upon.

Results. — As is seen in Table I the local administration of glucose in most cases elicited only a slight to moderate motility in jejunum. The SP content of the first piece of gut, taken prior to glucose injection, varied between 9.6 and 37 units per g. The SP content of the second piece of gut did not differ significantly from the first one. In one case (case 1) a lively motility was observed and in this case the SP content of the second piece was 35.0 units per mg compared to 23,6 in the initial one.

TABLE I

EFFECT OF LOCAL GLUCOSE ADMINISTRATION ON INTESTINAL MOTILITY AND SP CONTENT IN PATIENTS WITH DUODENAL ULCER.

Case	Initial P conc. (U./g tissue)	After local administration of 150 ml 50% glucose	
		Motility	SP
1	23.6	Lively	35.0
2	11.7	Slight	10.5
3	13.2	„	8.5
4	37.0	„	30.0
5	16.0	Moderate	8.0
6	26.0	„	28.0
7	9.6	Slight	9.3
11	12.9	„	6.2
12	14.8	„	12.6
13	15.1	Moderate	11.1

In the dumping series (Table II) the local glucose administration initiated in most cases a lively or very lively motility. In those cases a significantly higher SP concentration was obtained in the piece of gut taken after the stimulation as compared to the initial value.

TABLE II

EFFECT OF LOCAL GLUCOSE ADMINISTRATION ON INTESTINAL MOTILITY AND SP CONTENT IN PATIENTS WITH DUMPING SYNDROME.

Case	Initial P conc. (U./g tissue)	After local administration of 150 ml 50% glucose	
		Motility	SP
8	19.7	slight	17.7
9	6.9	very lively	19.8
10	10.6	very lively	13.3
14	4.1	moderate	10.5
15	4.8	very lively	16.9
16	18.4	slight	18.7
17	4.8	very lively	8.0
18	1.3	lively	2.4

All data hitherto obtained are summarized in Fig. 1, where the difference in SP concentration between the second and first pieces of gut is related to the effect in motility obtained by glucose administration.

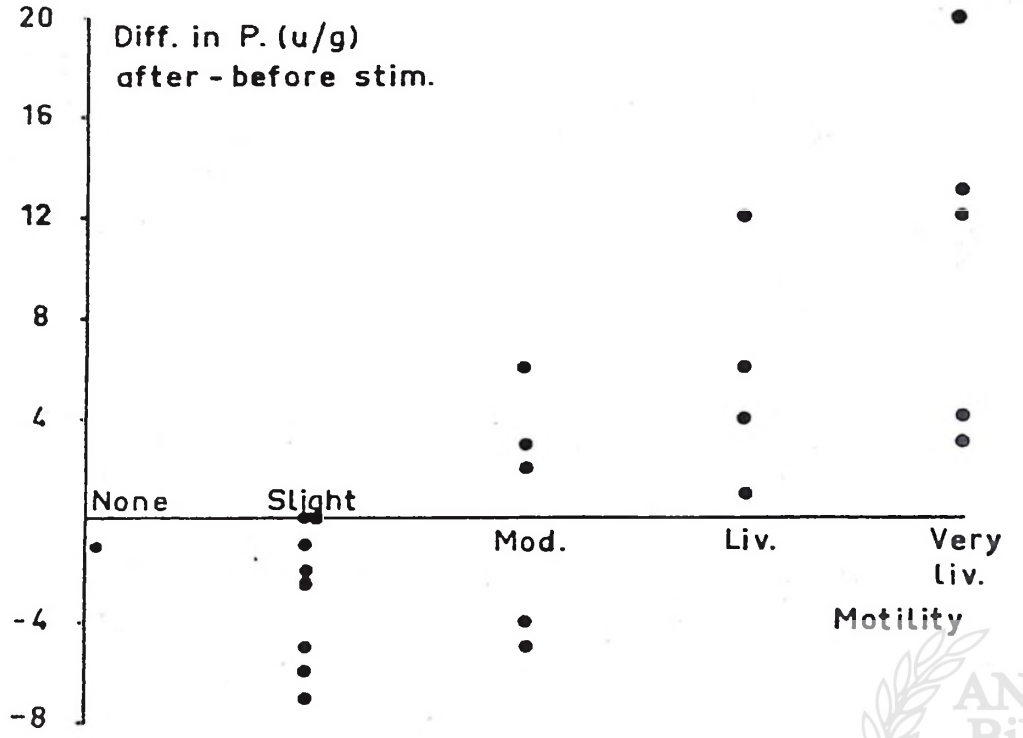


FIG. 1

Difference in substance P concentration (units per g wet tissue) between pieces of human jejunum taken after and before administration of glucose in relation to the degree of motility elicited by the stimulation.

Summary

The substance P content of the intestinal wall in man was studied before and after intraluminar administration of hypertonic glucose. No difference in substance P was obtained in those cases where only a slight increase in the motility was elicited by glucose. In those patients in which the glucose administration caused a marked increase in motility the substance P content of the intestine was higher after glucose then before.

ODNOS IZMEĐU SP I POKRETLJIVOSTI TANKOG CRIJEVA

Proučavao se sadržaj SP u stijenci čovjekovog crijeva prije i poslije intraluminalne aplikacije hipertoničnog rastvora glukoze. U onim slučajevima kad je glukoza izazivala samo slabo pojačanje pokretljivosti nisu zapažene razlike u sadržaju SP. Međutim, kod pacijenata kod kojih je aplikacija glukoze prouzrokovala znatno pojačanje motiliteta nađen je znatniji sadržaj SP poslije primjene glukoze nego prije nje.

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DISCUSSION

HUKOVIĆ: Have you taken the two pieces of gut at the same time, or have you always taken them after a certain time interval?

PERNOW: I always took them after a time interval.

ZETLER: Does the increase in SP concentration mean that the active principle is either synthesised faster or destroyed more slowly? Do you have any idea about the biochemical mechanism which leads to the change in SP concentration you observed?

PERNOW: Yes, I think an increase in SP concentration is best explained as either a faster synthesis or a slower rate of destruction. But I have no idea which explanation is relevant here.

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D. B. BELESLIN AND B. Ž. RADMANOVIĆ

THE EFFECT OF SUBSTANCE P ON THE ISOLATED SUPERIOR CERVICAL GANGLION OF THE CAT

It has been shown that SP, injected intra-arterially into the central end of the lingual artery while occluding the external carotid artery. potentiated the response of the nictitating membrane to submaximal stimulation of the preganglionic sympathetic nerve, while higher doses usually depressed the response. The stimulating action of ACh on the superior cervical ganglion »in situ« was also potentiated by SP (Beleslin, Radmanović and Varagić, 1960).

It was therefore of interest to reinvestigate the action of SP on the isolated superior cervical ganglion of the cat and to test whether changes in the output of ACh from the ganglion contribute to the failure of transmission.

Methods

Cats of both sexes, weighing 1.5 to 3 kg, were used. Anaesthesia in cats was induced with ether and maintained by intravenous injection of chloralose (100 mg/kg b. w.).

The superior cervical ganglion was prepared for perfusion following the conventional method, with the modifications suggested by Perry (1953). The contractions of the nictitating membrane were recorded with an isotonic lever fitted with a frontal writing point magnifying the movements of the membrane ten times. The cervical sympathetic chain was divided and, when stimulated electrically, its peripheral end was placed on shielded electrodes and covered with liquid paraffin. For stimulation an electronic stimulator delivering square wave pulses was used. The pulses had a duration of 0.8 msec. and a frequency between 2 and 5 per sec.

When ACh was to be collected, the post-ganglionic trunk was tied and eserine-salicylate, 10^{-6} g/ml, added to Locke's solution. The preganglionic nerve was stimulated intermittently, for 5 min. periods of stimulation, alternating with an equal period of rest. This procedure usually gave a series of samples containing an approximately equal amount of ACh. Five or six samples were usually collected.

ACh was assayed on the blood pressure of eviscerated, chloralosed cats.

SP was kindly supplied by Professor Gaddum, Dr. Lembeck and Dr. Pernow. SP supplied by Professor Gaddum and Dr. Pernow was a concentrate and contained 75 U./mg. SP supplied by Dr. Lembeck contained 5.6 U./mg.

Results

SP and sympathetic nervous stimulation. — Upon addition of SP in doses from 0.25 to 40 units to the fluid, perfusing preparations of the superior cervical ganglion, the changes in response of the nictitating membrane to submaximal stimulation of the preganglionic sympathetic nerve were recorded. In present experiments, when SP was added to the perfusing fluid, the contractions of the nictitating membrane in response to preganglionic nerve stimulation were usually depressed or blocked. Fig. 1 shows the responses of the nictitating membrane to stimulation of the cervical sympathetic trunk with stimulation periods of 5 sec. every 90 sec. After addition of 1 and 3 units of SP at P_1 and P_2 the response to preganglionic stimulation was unchanged. The responses of the nictitating membrane to preganglionic nerve stimulation at P_3 were slightly reduced after addition of 10 units of SP to the perfusing fluid. When 30 units of SP were added to the perfusing fluid (at P_4) the responses to preganglionic stimulation were completely abolished. The complete block lasted 12 min., after which the responses gradually returned to normal. The amount of SP which was added to the perfusing fluid did not, in itself, stimulate the ganglion, and caused no contraction of the nictitating membrane in the absence of preganglionic stimulation. The transient depression of the response of the nictitating membrane to preganglionic nerve stimulation was obtained even after small doses of SP. A typical experiment is shown in Fig. 2. At **P**, 0.5 units of SP was added to the perfusing fluid. The response to preganglionic stimulation was first reduced and after 15 min. completely abolished. The complete block lasted 5 min., after which the response gradually returned to normal. This type of response was obtained in 6 out of 9 experiments. The higher doses of SP (from 10 to 40 units) produced the block of the response to preganglionic nerve stimulation in 7 out of 10 experiments.

In present experiments the potentiation of the response of the nictitating membrane to preganglionic nerve stimulation was rarely observed after adding SP. After small doses of SP (from 0.25—2 units) its potentiating effect was observed only in 1 out of 9 experiments. Similar results were obtained with higher doses (from 10—40 units). This effect was observed only in 1 out of 10 experiments.

SP and ACh. — In present experiments, when SP was added to the perfusing fluid, the contractions of the nictitating membrane to ACh were depressed or blocked. A typical experiment is shown in Fig. 3. At **P** in **A**, 2 units of SP were added to the perfusing fluid

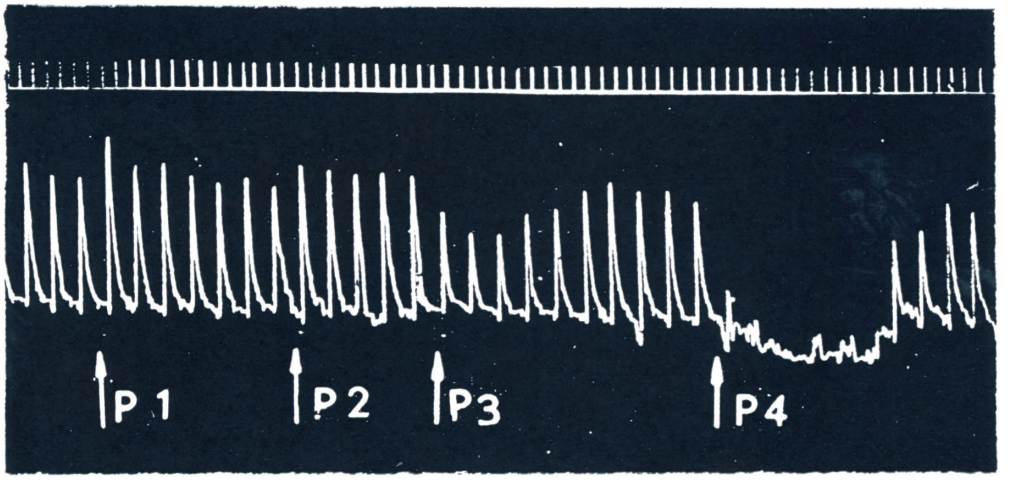


FIG. 1

Cat, chloralose. Contractions of the nictitating membrane to submaximal preganglionic stimulation of the cervical sympathetic nerve (5 pulses/sec., 0.8 msec., 7.5 mA). At P_1, P_2, P_3, and P_4 SP was added to perfusing fluid in doses of 1, 3, 10 and 30 units respectively. Time 1 min.

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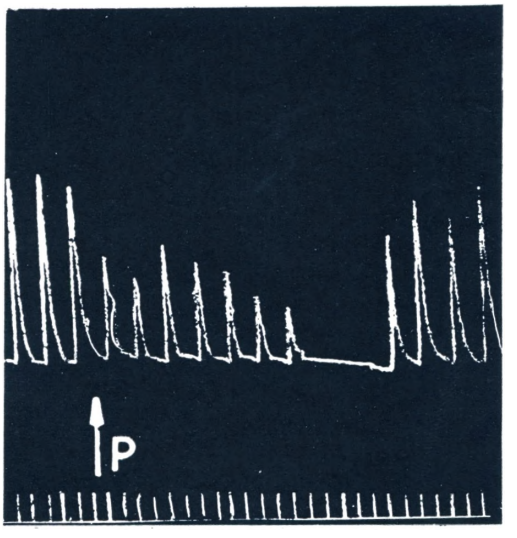


FIG. 2

Cat, chloralose. Contractions of the nictitating membrane to submaximal preganglionic stimulation of the cervical sympathetic nerve (5 pulses/sec., 0.8 msec., 7.5 mA). At 0.5 unit of SP was added to perfusing fluid. Time 1 min.

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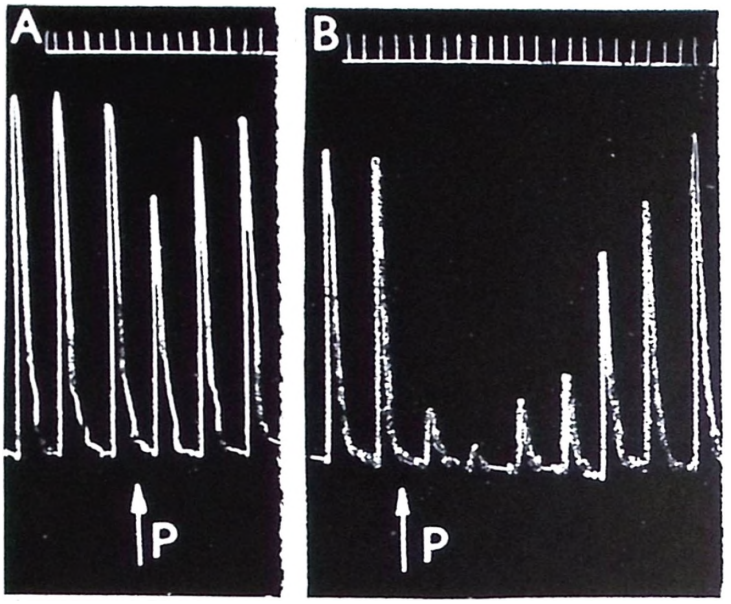


FIG. 3

Cat. chloralose. Contractions of the nictitating membrane to ACh (4 μg). At P in A, 2 units of SP was added to perfusing fluid. At P in B, 30 units of SP was added to perfusing fluid. Time, 1 min.

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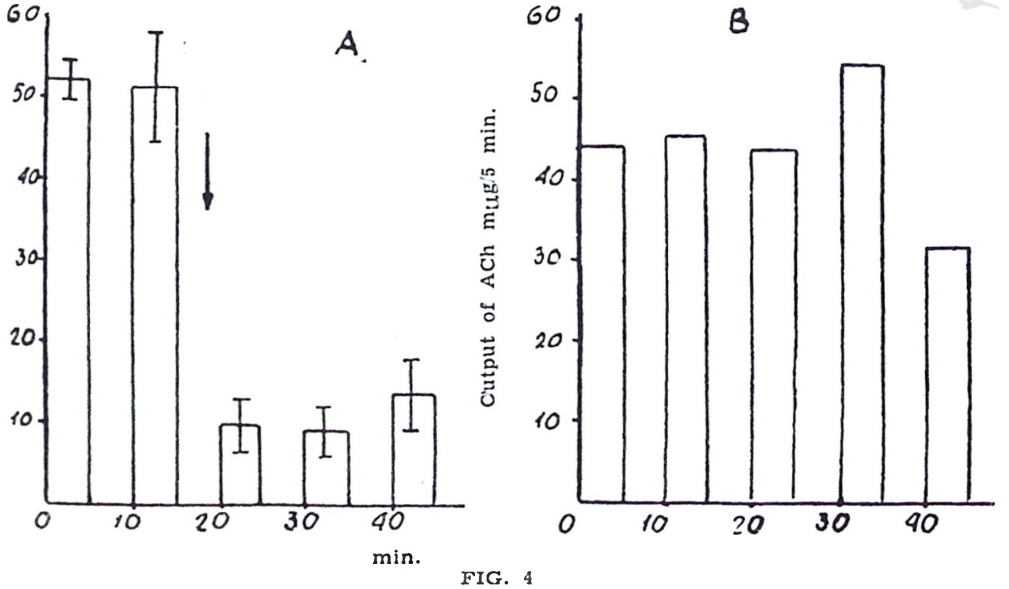


FIG. 4

Each block represents amount of ACh released during 5 min. stimulation of the cervical sympathetic nerve at 2/sec., 0.8 msec., 7.5 mA. At the arrow in A 20 units of SP was added to perfusing eserinized Locke's solution. In B the ganglion was perfused only with eserinized Locke's solution (no SP was added).

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and produced a transient depression of the response to ACh. 'The higher dose of SP (30 units) in the same experiments at **P** in **B** produced complete block of the response of the nictitating membrane to ACh. The depression of the response of the nictitating membrane after small doses of SP (from 0.1 to 5 units) was observed in 8 out of 11 experiments. The higher doses of SP (from 10—30 units) usually produced the block of the response to ACh. This was observed in 3 out of 4 experiments.

The potentiation of the response of the nictitating membrane to ACh after small doses of SP (from 0.1 to 5 units) was observed in 2 out of 11 experiments, and only in 1 out of 4 experiments after a higher dose of SP (30 units).

The effect of SP on ACh output. — Hutter and Krista Kostial (1954) found that, when the preganglionic nerve was stimulated intermittently for 5 min. periods of stimulation alternating with equal periods of rest, gave a series of samples containing an approximately equal amount of ACh. These experiments prompted us to test the effect of SP on ACh output. Using the same procedure we found that SP in a dose of 20 units decreased the ACh output. At the arrow in Fig. 4, 20 units of SP was added to the perfusion fluid. The ACh output was reduced by about 80%. This reduction lasted 30 min.

Discussion

These experiments show that SP, when added to the perfusing fluid, mostly depressed, and rarely potentiated, the transmission in the superior cervical ganglion. The depression was usually observed after lower as well as after higher doses of SP. We have found in these experiments that the failure of ganglionic transmission produced by SP is accompanied by a decrease in the amount of ACh liberated as a result of preganglionic stimulation.

The effect of ACh on the superior cervical ganglion was usually depressed. Higher doses of SP usually depressed the response of the nictitating membrane to ACh.

In our present experiments there was no difference in action on the superior cervical ganglion of the cat between the concentrated SP, supplied by Prof. Gaddum and Dr. Pernow, and SP supplied by Dr. Lembeck.

We have found that SP reduced the output of ACh from nerve endings. The extent to which this action contributes to the failure of transmission cannot be determined from our experiments. Brown and Feldberg (1936) found that the amount of ACh, liberated by a series of maximal preganglionic volleys, is in excess of the minimum required to produce full excitation of the ganglion cells. The reduction of responses to ACh also suggests that some other mechanism might be involved in the action of SP. The decreased output of ACh may also be due to ACh synthesis or to a cholinesterase activity of SP.

V. VARAGIĆ, D. B. BELESLIN AND B. Ž. RADMANOVIĆ

SOME PERIPHERAL NEUROTROPIC EFFECTS OF SUBSTANCE P

The potent smooth muscle stimulating effect of SP was first described by Euler and Gaddum (1931) and later studied in detail by Pernow (1953). Meanwhile, SP is also a normal constituent of brain tissue and has been found to produce various effects on the central nervous system [Euler and Pernow (1954), Zetler (1960)]. Lembeck (1957) presented evidence that SP stimulated afferent nervous fibres. It was therefore decided to study peripheral neurotropic effects of SP.

SP and peristalsis. — During the investigation of the effect of cooling and of 5-HT on the peristaltic reflex of the isolated guinea pig ileum (Beleslin and Varagić, 1958a) it was decided to test the action of SP on the peristalsis. In order to mimic physiological conditions as closely as possible, SP was injected intraluminally. For this reason the original Trendelenburg's method (1917) for recording the peristaltic activity was slightly modified, in order to allow the intraluminal injections. It was found that the introduction of SP into the lumen of the guinea pig ileum caused an increase in the number and amplitude of the peristaltic waves. SP was also found to be able to restore peristalsis in preparations, in which it was abolished by fatigue (Fig. 1), by external or internal application of 5-HT (Fig. 2), or by lowering the temperature of the bath (Beleslin and Varagić, 1958b). Since the effect of SP was absent in preparations in which the mucous membrane was removed, and since hexamethonium abolished the effect of SP on peristalsis (Fig. 3), it was concluded that SP acted on the afferent nervous elements of the peristaltic reflex arc possibly through the sensory receptors in the mucosa.

In experiments, in which the antagonism of morphine-like analgesics and SP on the peristaltic reflex was studied, Medaković and Radmanović (1959) have found that the stimulating action of SP on the peristaltic reflex was inhibited by morphine and morphine-like analgesics (Fig. 4). The relative inhibitory potency of analgesics on the stimulating action of SP corresponded well to their relative analgesic potencies. On the other hand, the intraluminally introduced SP could not restore the peristaltic reflex previously abolished by morphine and morphine-like analgesics (Fig. 5). Medaković (1959) has also found that

In our previous work (Beleslin, Radmanović and Varagić, 1960) a potentiating effect of SP was observed in 5 out of 11 experiments, when it was injected intra-arterially into the central end of the lingual artery, and a depression was obtained in 4 out of 11 experiments.

It is very difficult to explain the mechanism by which SP potentiates the responses of the nictitating membrane to the preganglionic nerve stimulation or to ACh. SP probably acts by sensitisation of ACh receptors in the postganglionic neuron, or this effect is due to nonspecific action of impurities in SP itself. In our previous work we used unpurified SP (obtained by extraction of ox brain). This preparation contained usually about 2—3 U./mg. The potentiating effect of SP on the ganglion »in situ« might be connected with the presence of blood. The greater percentage of potentiation on the ganglion »in situ« observed in our previous experiments might also be due to the presence of some other active substances in unpurified SP.

Summary

(1) SP added to the perfusing fluid in lower and higher (0.25—40 units) doses usually depressed the response of the nictitating membrane to preganglionic sympathetic nerve stimulation.

(2) The stimulating action of ACh on the superior cervical ganglion, as judged by contraction of the nictitating membrane, was also depressed after adding SP to the perfusing fluid.

(3) SP in a dose of 20 units, which usually produced block of ganglionic transmission, reduced at the same time the output of ACh.

EFEKAT SP NA IZOLOVANI GORNJI VRATNI GANGLION MAČKE

Dodatak SP perfuzionoj tekućini u dozama 0.25—40 jed. obično smanjuju reakciju membranae nictitantis na preganglionarnu simpatičku stimulaciju.

Stimulativno djelovanje ACh na gornji vratni ganglion također je smanjeno poslije dodatka SP, sudeći po kontrakciji membranae nictitantis.

SP u dozi od 20 jed., koja je dovoljna da izazove blokadu ganglionarne transmisije, ujedno snižava izlučivanje ACh.

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DISCUSSION

LEMBECK: The inhibition of the ganglion stimulation by SP seems to be an effect which does not appear immediately after the injection (like hexamethonium), but shows a slow onset and exerts its full effect not earlier than after some minutes.

KRIVOY: The original observation of Beleslin *et al.* that SP potentiates ganglion transmission have been repeated in my laboratories using electrophysiological techniques *in vivo* and *in vitro*, although the dose of SP was much smaller (20 i. U./ml).

morphine, acting from the intestinal lumen, can paralyse the peristaltic reflex, possibly by acting on the sensory mucosal receptors. It was therefore concluded that morphine and SP acted at the same point. The surmountability in the interaction of SP and analgesics was not demonstrated in these experiments.

SP acting on the outside of the guinea pig ileum. — Large doses of SP (30—100 units), when acting on the outside of the isolated guinea pig ileum, abolished the peristaltic reflex, whereas small doses (4 units) occasionally potentiated peristalsis (Beleslin and Varagić, 1959a). This block could not be overcome by intraluminal injections of 5-HT or itself (Fig. 6). It was also found on the Magnus preparation that a high dose of SP can block the response of the gut to a small dose of the same substance.

Recently Radmanović (1961, in press) has found that small doses of SP when acting on the outside of the isolated guinea pig ileum could restore the peristaltic reflex previously abolished by *d*-tubocurarine (*d*-TC) (Fig. 7). On the other hand, previous addition of SP into the bath did not abolish the inhibitory effect of *d*-TC on the peristalsis. In similar circumstances 5-HT injected intraluminally failed to restore peristalsis which was abolished by *d*-TC. This effect of SP was attributed to its action on the nervous elements in the gut, possibly on the intestinal ganglia, because *d*-TC was found to have a ganglion blocking activity [Feldberg and Lin (1949), Paton and Zaimis (1949)].

SP and the amount of 5-HT in the gut. — 5-HT was known to affect peristalsis in a similar way as SP. It stimulates peristalsis

TABLE I

THE EFFECT OF SP ON THE AMOUNT OF 5-HT IN THE ILEUM, STOMACH AND SPLEEN OF THE RAT (BELESLIN AND VARAGIĆ, 1959b)

Number of experiments	Ileum (µg 5-HT/g)		P	Stomach (µg 5-HT/g)		P	Spleen (µg 5-HT/g)		P
	Controls	SP, 100 U./kg		Controls	SP, 100 U./kg		Controls	SP, 100 U./kg	
1	1130	1574		1126	2086		1871	2307	
2	1303	2742		1598	1860		1000	1498	
3	1215	1670		1056	1452		714	1918	
4	754	1336		1553	1558		857	1428	
5	720	2218		988	1684		1124	615	
6	826	1578		891	2072		1076	1198	
7	340	1800		1919	2374		1810	1995	
8	902	1460		711	1743		1082	1018	
9	1480	1572		951	2198		972	1047	
10				1694	2308		705	1869	
Mean	964	1773		1249	1933		1121	1489	
			0.01			0.01			0.1
st. error	118.1	146.1		119.9	96.2		128.4	191.8	

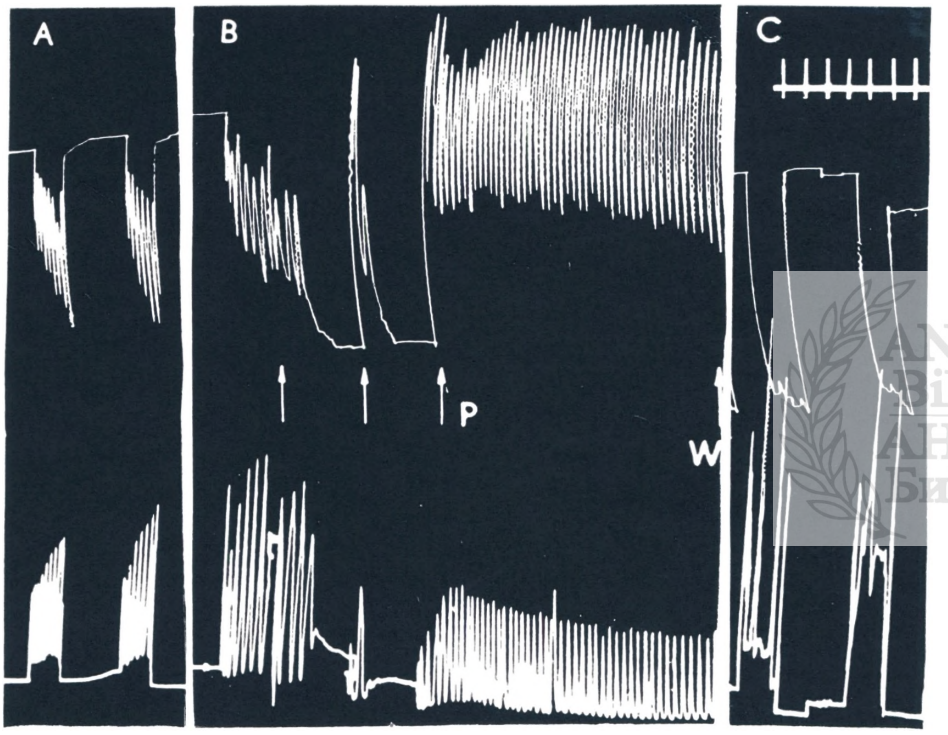


FIG. 1

The effect of SP on peristalsis in fatigued guinea pig ileum. **A**, normal peristaltic response to a raised intraluminal pressure. **B**, at first arrow, 0.2 ml Tyrode intraluminally (i. l.); at. **P**, 10 U. SP i. l.; at **W**, bath and intestine washed out. **C**, 110 min. after washing. Upper record: peristalsis. Lower record: contractions of longitudinal muscle. Time, 1 min. (Beleslin and Varagić, 1958).

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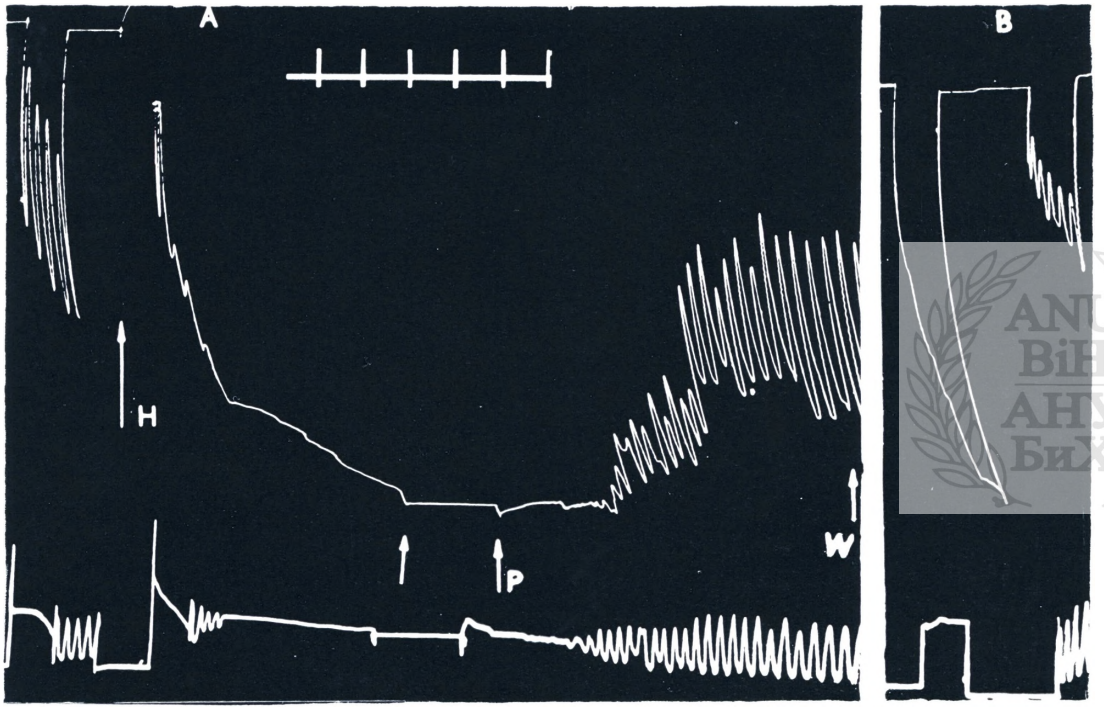


FIG. 2

Restoration of peristalsis by SP after inhibition by 5-HT. Upper record: peristalsis. Lower record: contractions of longitudinal muscle. **A**, at **H**, 1 mg 5-HT added to bath; at arrow, 0.2 ml Tyrode intraluminally (i. l.); at **P**, 10 U. SP i. l.; **W**, bath and intestine washed out. **B**, peristalsis 25 and 140 min. after washing out. Time, 1 min. (Beleslin and Varagić, 1958).

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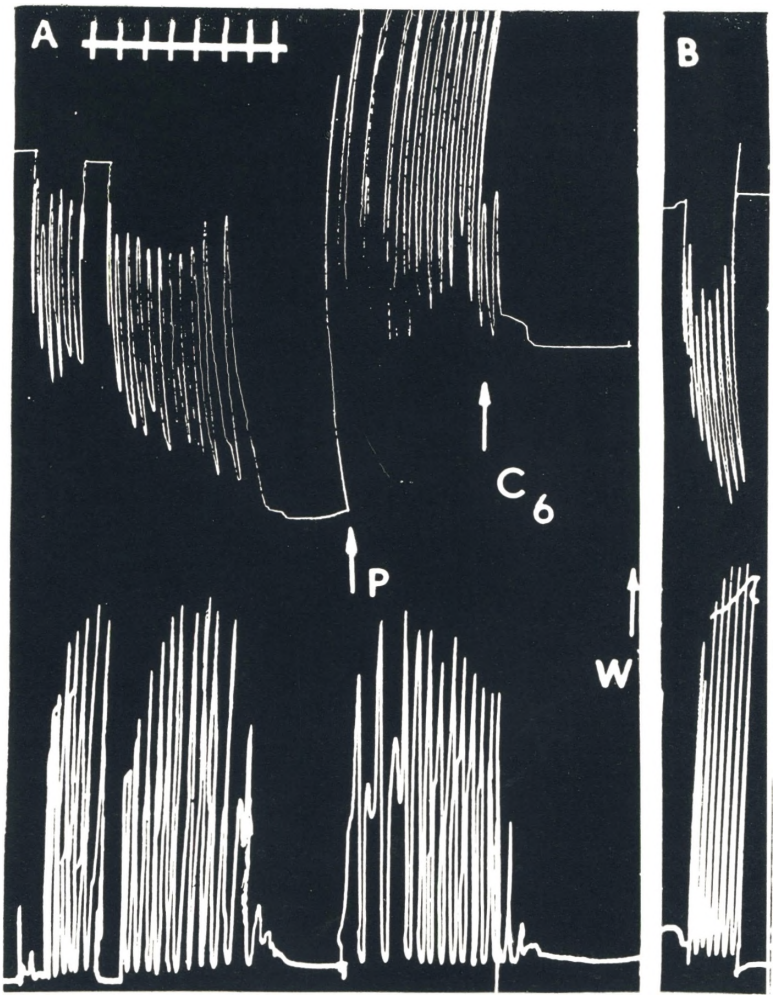


FIG. 3

The effect of hexamethonium (C_6) on peristalsis produced by SP. Upper record: peristalsis. Lower record: contractions of longitudinal muscle. **A**, at **P** 10 U. SP i. l.; at C_6, 0.4 mg hexamethonium added to bath; at **W**, bath and intestine washed out. **B**, 45 min. after washing. Time, 1 min. (Beleslin and Varagić, 1958b).

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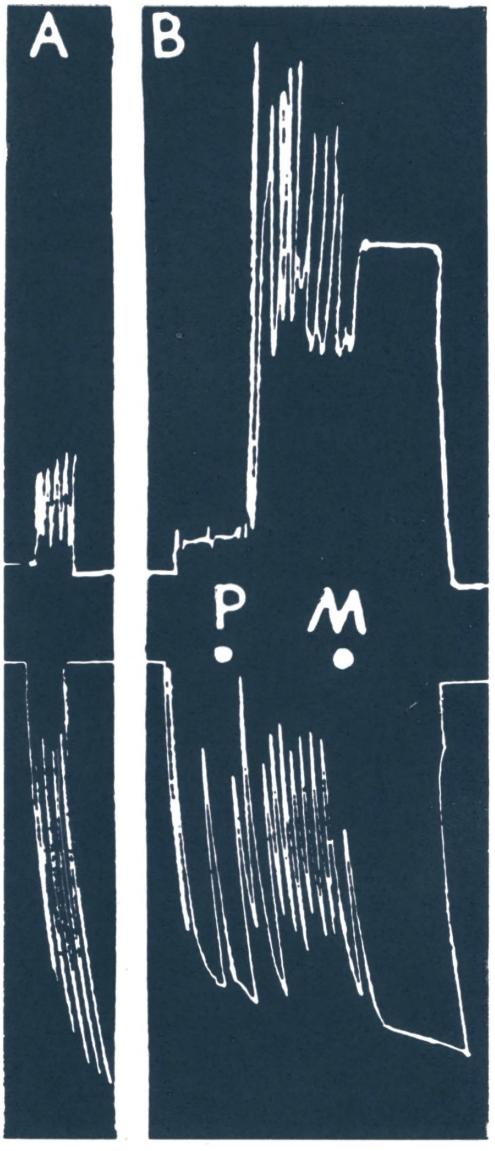


FIG. 4

The effect of morphine on peristalsis produced by SP. Upper record: contractions of longitudinal muscle. Lower record: peristalsis. **A**, control record. **B**, at **P** 20 U. SP i. l.; at **M**, 1 µg morphine i. l. (Medaković and Radmanović, 1959).

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FIG. 5

The effect of SP on peristaltic block produced by morphine. Upper record: contractions of longitudinal muscle. Lower record: peristalsis. **A**, control record. **B**, at **P** 20 U. SP i. l. Between **A** and **B** 1 μg morphine i. l. (Medaković and Radmanović, 1959).

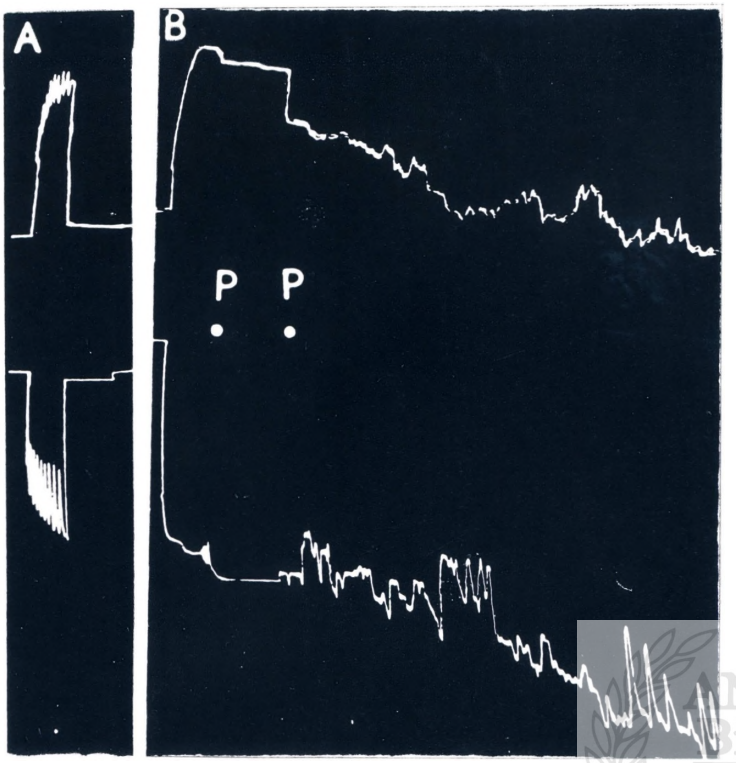
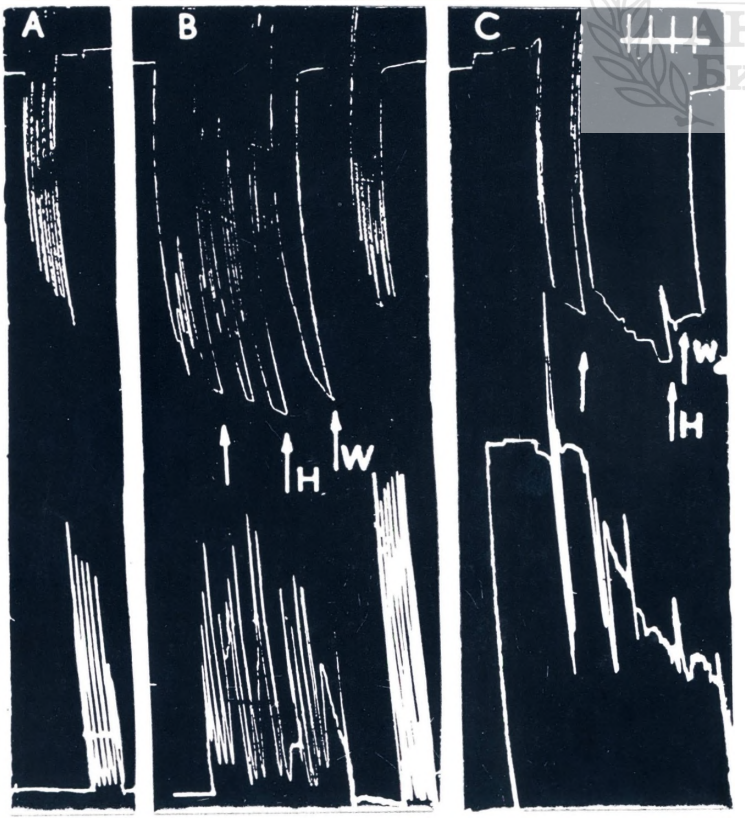


FIG. 6

The effect of 5-HT on the peristaltic block produced by SP. Upper record: peristalsis. Lower record: contractions of longitudinal muscle. **A**, control record. Between **A** and **B**, 30 units and between **B** and **C**, 50 units of SP added to the bath. At the arrow, 0.2 ml Tyrode i. l.; at **H**, 2 μg 5-HT i. l.; at **W**, bath and intestine washed out. Time, 1 min. (Beleslin and Varagić, 1959a).



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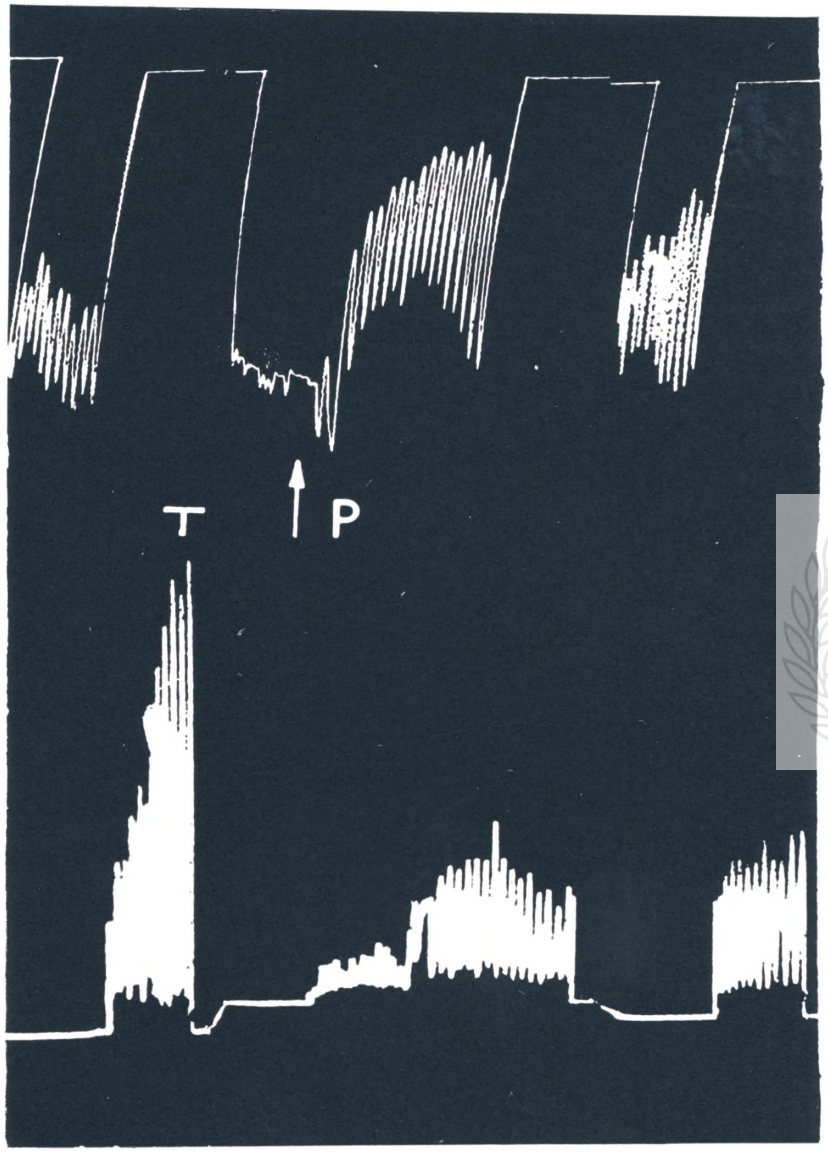


FIG. 7

The effect of SP on the peristaltic block produced by d-TC. Upper record: peristalsis. Lower record: contractions of longitudinal muscle. At **T**, 0,25 mg d-TC added to the bath. At **P**, 4 U. SP added to the bath. Bath volume: 15 ml.

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when acting on the mucous membrane of the intestine (Bülbring and Lin, 1957) and it can depress or abolish the peristaltic reflex when acting on the outside of the isolated intestine [Kosterlitz and Robinson (1957), Ginzel (1957)]. Considering this similarity of action of SP and 5-HT on the peristaltic reflex, experiments were carried out in order to see, whether SP can alter the 5-HT content of the gut. It was found that SP increased the amount of 5-HT both in the isolated guinea pig ileum and rat ileum (Table I). The same was true for the rat stomach. On the contrary, no change in 5-HT content of the rat spleen was observed after treatment of the animal by 100 U./kg intraperitoneally for three successive days. It is remarkable that SP increased the amount of 5-HT only in organs containing chromaffine tissue (Beleslin and Varagić, 1959b).

Interaction with other biologically active substances. — It was found in the isolated guinea pig ileum that SP potentiated the responses to nicotine and ACh and depressed the effect of 5-HT. The responses to histamine were unaffected by SP (Beleslin and Varagić, 1960). It has been known that blocking the ganglia by hexamethonium slightly reduced the response to ACh in a number of experiments on the isolated guinea pig ileum (Feldberg, 1951). Therefore, at least in this preparation, ganglion stimulation contributed to a certain extent to the ACh contraction of the intestine. In accordance with this view it was found that the potentiating effect of SP on the response to ACh was abolished by hexamethonium (Fig. 8). SP potentiated also the response of the guinea pig ileum to nicotine (Fig. 9). It is generally accepted that both ACh and nicotine depolarize the ganglion cells, but our experiments do not allow any conclusion regarding the mode of action of SP on the process of depolarization.

Similarly to SP, 5-HT was found to increase the longitudinal muscle contractions in response to ACh while the ganglionic effect of nicotine was first facilitated and then blocked (Bülbring and Crema, 1958). SP in large doses was also found to depress the response to nicotine (Fig. 9). On the other hand SP depressed the response to 5-HT, but it is not sure whether muscular or nervous 5-HT receptors were affected (Gaddum and Picarelli, 1957). Feldberg (1951) has shown that there was no evidence that the histamine (H) contractions of the guinea pig ileum were, in part, the result of stimulation of nerve endings. Beleslin and Varagić (1960) have found that SP did not change the response of the guinea pig ileum to H. It is therefore possible that SP potentiated the responses of the guinea pig ileum only to those biologically active substances which act completely or partly through the nervous structures.

It has also been shown that the response of the longitudinal muscle of rabbit and guinea pig intestine to SP is partly depressed by lowering the temperature of the bath. It is known that the nervous structures are more thermo-sensitive than the muscle fibres and this has also been taken as an evidence for the neurotropic effect of SP (Pernow,

1960). The previous evidence presented by Pernow (1953) suggested that SP stimulates the muscle fibres directly. To these findings the peripheral neurotropic effects of SP are now added.

Radmanović (1961b, in press) has found that *d*-TC and gallamine inhibit the longitudinal muscle contractions of the isolated guinea pig ileum caused by SP. The doses of *d*-TC and gallamine producing 50 per cent inhibition (ED_{50}) of the effect of SP were 0.0018 μg/ml and 0.035 μg/ml respectively. A dose-effect relationship was found in the inhibitory action of *d*-TC toward the SP. On the other hand, succinylcholine was found to potentiate slightly the effect of SP.

SP and the superior cervical ganglion. — While investigating peripheral neurotropic effects of SP some evidence was obtained indicating that block of peristalsis might be, at least partly, produced by the action of SP on the intestinal ganglia. It was therefore decided to investigate this problem in detail. It was found that the intraarterial injections of SP in doses from 10 to 30 units potentiated the response of the nictitating membrane of the cat to submaximal stimulation of the preganglionic sympathetic nerve (Fig. 10), while higher doses (30—100 U.) usually depressed the response (Fig. 11). The stimulating action of ACh on the superior cervical ganglion (as judged by the response of the nictitating membrane) was also potentiated by SP. It was found that that the responses of the nictitating membrane to adrenaline, noradrenaline and tyramine were potentiated by SP as well (Beleslin, Radmanović and Varagić, 1960). All these results were obtained on the superior cervical ganglion »in situ«.

Beleslin and Radmanović (unpublished results) repeated these experiments on the isolated superior cervical ganglion and found more frequently a depressive action of SP. It is possible that the excitatory action of SP on the ganglion »in situ« might be connected with the presence of blood. Some interaction of SP and blood constituents might take place before it acts on the ganglion cells. Beleslin and Radmanović carried out their experiments with a highly purified sample of SP, while the previous experiments were done with unpurified batches of SP. It is possible that in the raw material containing SP some other active substance might be present.

Summary

The ability of SP to stimulate smooth muscle has been described as far back as 1931 (Euler and Gaddum), but its more detailed pharmacological study was carried out only in 1953 (Pernow). This substance is, however, a normal constituent of brain tissue, and was found to exert a stimulating activity in aferrent neurons. It was, therefore, decided to study in more detail the peripheric neurotropic effects of SP.

SP strongly stimulated peristaltic movements in the isolated guinea pig ileum (Beleslin and Varagić, 1958b). So it was possible, e. g., to attain the resuming of peristalsis by intraluminal injections into pieces

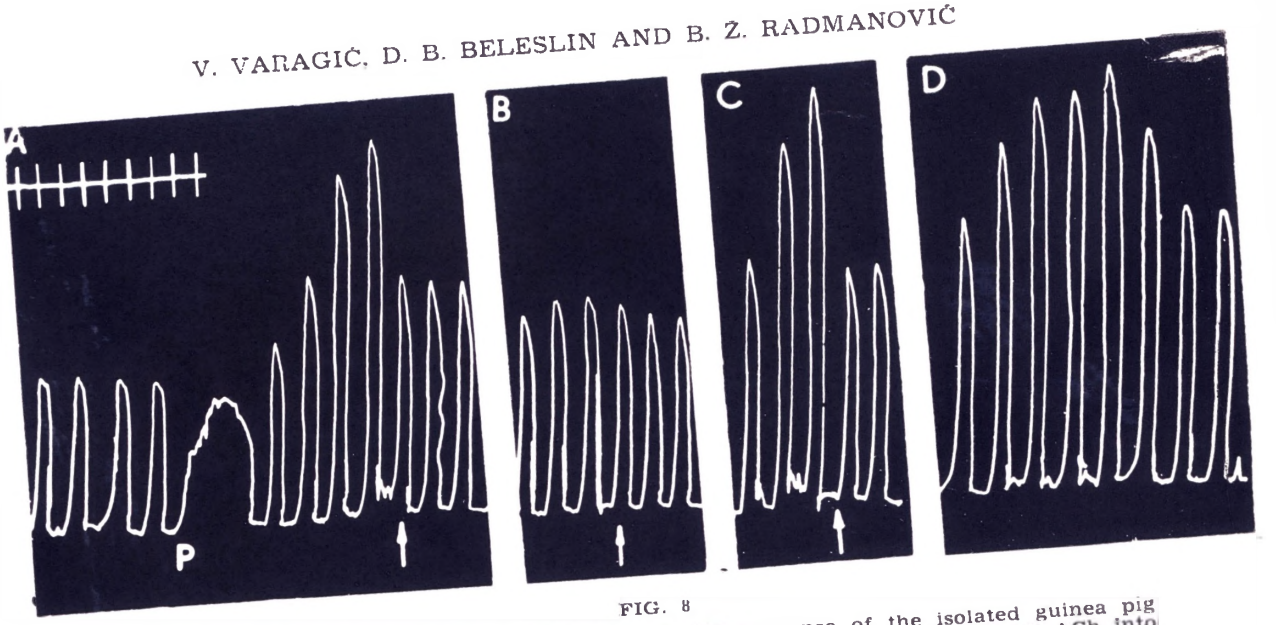


FIG. 8

The effect of SP and of hexamethonium on the response of the isolated guinea pig ileum to ACh. Contractions of the intestine were produced by adding 4 µg ACh into the bath every 4 min. At **P** in **A** and between **B** and **C** 3 units of SP added into the bath. At the arrows 5 µg hexamethonium added into the bath 2 min. before ACh. **B** was taken 40 min. after first addition of SP. **C** was taken 10 min. and **D** 30 min. after second addition of SP. Time, 1 min. (Beleslin and Varagić, 1960).

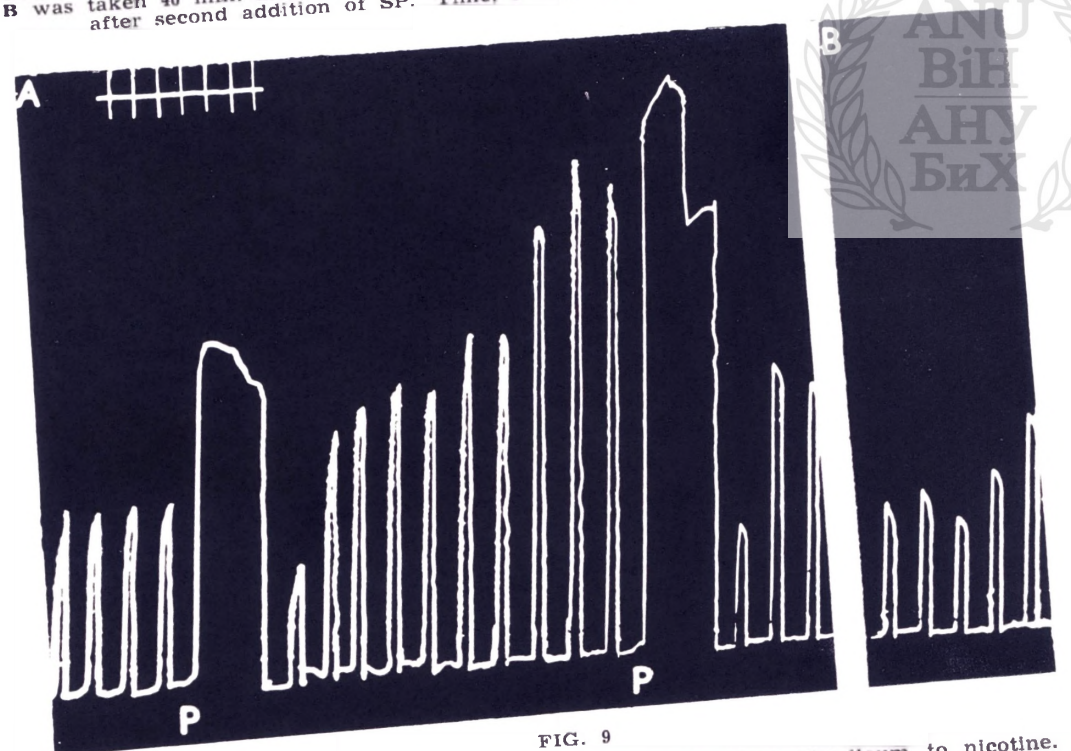


FIG. 9

The effect of SP on the response of the isolated guinea pig ileum to nicotine. Contractions of the intestine were produced by adding 1.5 µg nicotine into the bath every 10 min. At **P** 3 units and at P_1 30 units of SP into the bath. **B** was taken 30 min. after addition of 70 U. SP into the bath. Time, 1 min. (Beleslin and Varagić, 1960).

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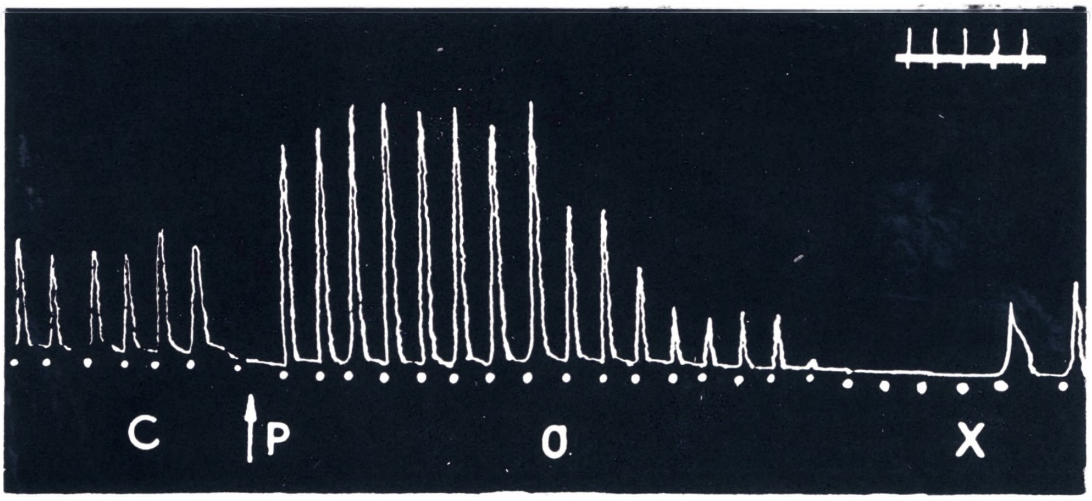


FIG. 10

The effect of SP on the superior cervical ganglion of the cat. Contractions of the nictitating membrane due to submaximal preganglionic stimulation of the cervical sympathetic nerve (5 pulses/sec., 0.8 msec., 7 mA, at dots) for 5 sec. every min. At **C** the external carotid artery was occluded. At **P**, 18 U. SP injected into the lingual artery. At **O** the external carotid artery was opened. At **X**, kymograph stopped for 15 min. Time, 1 min. (Beleslin, Radmanović and Varagić, 1960).

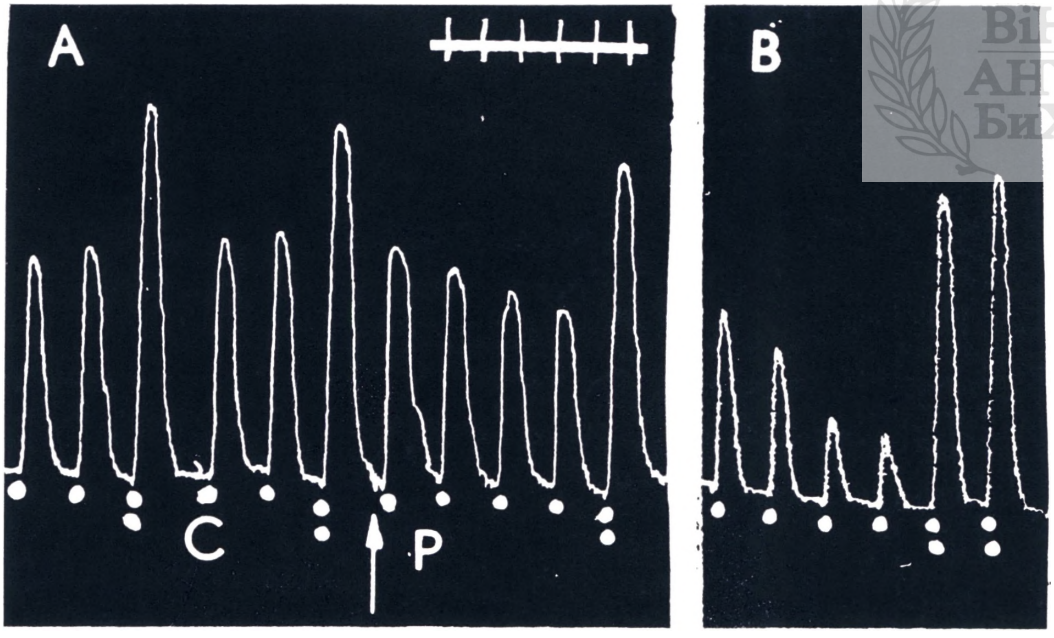


FIG. 11

Contractions of the nictitating membrane of the cat (at dots) due to submaximal preganglionic stimulation of the cervical sympathetic nerve (4 pulses/sec., 0.8 msec., 1.2 mA) for 10 sec. every 90 sec. At every two dots: submaximal postganglionic stimulation of the cervical sympathetic nerve (4 pulses/sec., 0.8 msec., 0.1 V) for 10 sec. every 90 sec. At **C** the external carotid artery was occluded. At **P** 100 U. SP injected into the lingual artery. **B** was taken 20 min. after injection of SP. Time, 1 min. (Beleslin, Radmanović and Varagić, 1960).

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of gut where peristaltic activity had been previously blocked by fatigue, action of 5-HT, or lowering of temperature. This effect of SP could not be suppressed by hexamethonium, hence it was concluded that its mechanism is a stimulation of afferent nerve elements in the peristaltic reflex arc.

The stimulating effect of SP on peristalsis was considerably weaker with outside treatment of the gut (Beleslin and Varagić, 1959a). In these experiments frequently even blocking of the peristaltic reflex was observed.

SP increases the 5-HT content in the isolated guinea pig ileum and in rat ileum. Intraperitoneal administration, in contrast, did not change the amount of 5-HT in the spleen (Beleslin and Varagić, 1959b). Strikingly, SP increases the 5-HT content in organs with chromaffine tissue only.

In the isolated gut SP potentiated the action of substances only, which partly or entirely act through nervous elements (e. g. ACh and nicotine), while it did not affect substances acting directly on smooth muscle (Beleslin and Varagić, 1960).

Intraarterial injections of SP potentiated the response of the nictitant membrane to submaximal stimulation of preganglionic fibres in the superior cervical ganglion of the cat in situ. Under the same conditions higher doses of SP depressed the response of the nictitant membrane (Beleslin, Radmanović and Varagić, 1960). In the isolated superior cervical ganglion of the cat Beleslin and Radmanović (This Symposium) found that SP exerts almost exclusively a depressive action.

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NEKI PERIFERNI NEUROTROPNI EFEKTI SUPSTANCIJE P

Sposobnost supstancije P (SP) da stimuliše glatku muskulaturu opisana je još 1931. godine (Euler i Gaddum), a farmakološki je detaljnije proučavana tek 1953 (Pernow). Ova supstancija je, medutim, i normalni sastojak moždanog tkiva, a nadeno je da ima i nadražajno delovanje na aferentna nervna vlakna. Iz tog razloga je bilo odlučeno da se detaljnije prouče periferni neurotropni efekti SP.

Nadeno je da SP snažno stimulira peristaltiku na zamorčevom izolovanom ileumu (Beleslin i Varagić, 1958b). Tako je intraluminalnim ubrizgavanjem SP bilo mogućno izazvati peristaltiku ako je ona bila blokirana za morom, 5-HT-om ili hladenjem. Ovaj efekat je bilo mogućno isključiti heksametonijumom. Zbog toga je zaključeno da SP draži aferentne nervne elemente peristaltičkog refleksnog luka.

Znatno slabiji nadražajni efekat na peristaltiku imala je SP kad je aplikovana spolja na crevo (Beleslin i Varagić, 1959a). Naprotiv, posle spoljašnje aplikacije SP, češće je dolazilo do bloka peristaltičkog refleksa.

SP povećava količinu 5-HT u zamorčevom izolovanom ileumu i u ileumu pacova. Suprotno ovome, SP aplikovana intraperitonealno ne menja količinu 5-HT u slezini (Beleslin i Varagić, 1959b). Pada u oči da SP povećava količinu 5-HT samo u organima koji sadrže hromafino tkivo.

Na izolovanom crevu SP potencira delovanje samo onih supstancija koje deluju delimično ili potpuno putem nervnih elemenata (na primer, ACh i nikotin), dok ne menja efekte materija koje deluju direktno na glatku muskulaturu (Beleslin i Varagić, 1960).

Na gornjem cervikalnom ganglionu mačke in situ nadeno je da intraarterijsko ubrizgavanje SP potencira reakciju membrane niktitans na submaksimalno draženje preganglijskih simpatičkih vlakana. Veće doze SP u istim uslovima deluju depresivno i smanjuju reakciju membrane niktitans na preganglijsko draženje (Beleslin, Radmanović i Varagić, 1960). Beleslin i Radmanović (v. sledeći članak) našli su skoro isključivo depresivno delovanje SP na izolovanu gangliju mačke.

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DISCUSSION

LEMBECK: The effect of SP on the intestinal HT content raises the question whether it means an increased synthesis or a decreased release of HT. It would be interesting to know the influence on the intestinal content.

RADMILA PRŽIĆ

THE INFLUENCE OF SUBSTANCE P ON ATTACK AGAINST THE »WRITHING SYNDROME« IN THE MOUSE

Stern and Huković (1960) have made a comparison between central and peripheral effects of SP using both a crude (5 U./mg) and a highly purified (270 U./mg) preparation. They concluded that periphereal effects change parallelly to the purity of the preparation but central effects do not. Zetler (1956) has reported several central effects of SP using crude preparations of a similar potency as that used in the present work. He found, inter alia, that SP protects the mouse from strychnine convulsions, lengthens the duration of hexobarbital narcosis and antagonizes the analgetic effect of morphine (Mo). Moreover, animals receiving SP are much more quiet than controls. These results are interpreted by Zetler on basis of the assumption that SP acts not only as a transmitter in sensory neurons as it was assumed by Lembeck (1953), but also in the neurons of inhibiting systems. For such reasons Stern and coll. were led to the conclusion that there exists a synergism between SP and central depressants (Mephenesin and Meprobamate), whereas no synergism exists between SP and autonomic depressants (Stern, Dobrić and Mitrović-Kocić, 1957).

Stern and Huković (1960) could not observe a potentiation of central effects by using a more purified preparation, at least not in all cases. Purified SP, e. g., does not protect the mouse against strychnine convulsions; on the contrary, the latter are enhanced. Likewise, there is no effect on hexobarbital narcosis. With respect to its antianalgesic action, however, crude SP shows almost equal effects as the purified preparation and antagonized Mo analgesia as reported by Zetler (1956). We have considered that the examination of the antianalgesic effect of SP by a different method would be useful in deciding whether this effect is an accidental one, or not. It is fully justified to put such a question since Fleisch (1953) has demonstrated before that an analgetic acts with different intensity, depending on the method for testing the sensation of pain. We were well aware, of course, that already Stern and Huković (1960) used a method different from that of Zetler. We have chosen the writhing of the mouse as a criterion, because the corresponding method produces visceral pain (Eckhardt, Cheplovitz,

Lipo and Govier, 1958). Apart form SP we have examined the effect of adenosine 5'-monophosphate (AMP) by the »writhing« method, because Laszlo (1960) has shown that AMP inhibits the periphereal effects of SP, and we wanted to see whether the same action could be observed with respect to the antianalgesic action. A further paper from our laboratory has established the fact that AMP protects against strychnine convulsions and lengthens the duration of hexobarbital narcosis, as well as analgesia produced by Mo (Huković, Košak and Stern, 1961).

Method

The method applied was that by Eckhardt and coll. (1958). Mice of both sexes, weighing 18 to 20 mg, were given a dose of 10—12 mg per animal SP i. p. (1 mg SP = 5 units), 1 mg/kg Mo s. c. and 100 mg/kg AMP s. c. Pain was provoked by 10 mg/kg HCl i. p. or, in some instances, by the histamine liberator L 1935, 2.5 mg/kg.

	SP	AMP*	
HCl	0/10	0,10	Remark
Mo-HCl	0/10	10/10	The quotient denotes the number of treated animals protected from pain vs. the number of treated animals
L 1935	0/10	0,10	
Mo-L 1935	0/10	10/10	

* AMP itself partly lowers the sense of pain.

Table I shows that SP is an antianalgesic antagonizing Mo with the method employed. In the controls HCl produced intense writhing and rubbing of the abdomen against the bottom of the cage. The application of Mo completely eliminated these symptoms. AMP could not break through Mo analgesia but reduced the pain due to HCl, in accordance with the findings of Huković, Košak and Stern (1961). So the antianalgesic effect observed with the SP preparation is an individual effect of SP itself. Consequently SP shows a marked central action, although it is unable to protect against strychnine convulsions or to lengthen hexobarbital narcosis. From another paper it will be seen that SP antagonizes harmine-induced tremor, an effect noticed already by Zetler, whilst AMP does not. Recalling Lembeck's (1953) finding much higher levels of SP in dorsal than in ventral roots, his subsequent work with the rabbit auricle, that by Holton (1960) and Serafimov (1959), and comparing with our own results about the antianalgesic effect of SP we may tell that all of this supports Lembeck's assumption that SP takes part in the transmission of sensory impulses. Further confirmation which is to be seen in the influence which light and darkness exert on the amount of SP in the retina (Stern and Kocić-Mitrović, 1958) and in the potentiation of strychnine convulsions by purified SP (Stern and Huković, 1960). It would seem that the rôle of SP as a transmitter in neurons of inhibitive systems is

less marked. It must be noted, however, that Stern and Huković (1961) observed an extraordinarily quieting effect of SP, purified over an alumina column and completely free from AMP, in mice. Thus a central sedative effect undoubtedly exists. It is most difficult, for the time being, to discuss the physiological significance of the anti-analgesic effect of SP. We do not know as yet, e. g., whether the same antagonism exists against other analgesics outside the Mo group. Pain, as a physiological phenomenon, is a defensive mechanism. So we can consider the potentiation of the sensation of pain, and the simultaneous potentation of reflex capacity (strengthening of strychnine convulsions), as serving the defense reactions of the organism.

In conclusion we can say that AMP is not responsible for the antianalgesic effect of SP preparations, and that this effect is reproducible by various methods.

Summary

SP is considered to act as the transmitting substance in sensory neurons. The antianalgetic effect of SP against morphine has been examined in the mouse writhing test as a specific test for sensation of visceral pain. Adenosine-5'-monophosphate has been examined in the same test in order to see whether it is capable to inhibit the central effect of SP as it was shown earlier to inhibit the peripheral effects of SP. Only SP acted as an antagonist to morphine analgesia, adenosine-5'-monophosphate did not act the same way. Hence, SP has a marked central effect.

UTJECAJ SUPSTANCIJE P NA NAPADAJ PROTIV »WRITHING« SINDROMA KOD MIŠA

Smatra se da SP djeluje kao transmitorna supstancija senzibilnih neurona. Ispitan je antianalgetski efekat SP u odnosu na morfin metodom svijanja miša kao specijalnim testom za osjet visceralnog bola. Istim testom ispitan je i efekat adenozin-5'-monofosfata (AMP) radi utvrdivanja eventualnih smetnji centralnog efekta SP, budući da je već ranije dokazano da AMP sprečava njene periferne efekte. Pokazalo se da samo SP djeluje antagonistički u odnosu na analgeziju izazvanu djelovanjem morfina, dok AMP ne djeluje na efekat morfina. SP, dakle, ima izrazit centralni efekt.

ACKNOWLEDGMENT. — Thanks are due to the Federal Council for Scientific Research (Yugoslavia) for financial help.

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DISCUSSION

KRIVOY: Drug-houses use the writhing method to test for antiemetics.

PRŽIĆ: The main point of this study is that SP antagonizes morphine.

ZETLER: How can one produce the writhing syndrom?

PRŽIĆ: By means of HCl histamine liberator L 1935, or with benzoquinone.

ANU BiH АНУ БиХ

R. MILIN

THE INFLUENCE OF SUBSTANCE P ON THE NERVOUS SYSTEM OF THE EARTH WORM (LUMBRICUS TERRESTRIS)

The results of pharmacological investigations of the action and distribution of SP in the nervous system indicate a peculiar importance of this factor in nerve activity (Gaddum, 1960). The depressing, i. e. inhibitory, effects of SP in particular parts of the nervous system (Zetler, 1960), as well as its markedly tranquillizing properties (Stern and Dobrić, 1957) have already been described in medical literature.

No indications, however, could be found in the various reports on SP that its presence had been sought for in the nervous system of the earth worm. In view of the simplicity of the earth worm's nervous system and its high sensitivity to various ecological factors we have undertaken a study of the influence of SP on this organism and propose to report in the present paper some of the results obtained with SP in cerebral ganglia and the subpharyngeal ganglion in the earth worm subject to agression.

Materials and methods

Adult earth worms of similar weight, collected in November from the same place and the same day, have been divided into four groups. Group I was kept constantly in the dark; Group II was exposed to daylight and to light of a 200-W bulb for 24 hours; Group III, again, was kept in the dark but was, at the same time, exposed to the action of SP; Group IV was exposed to both SP and light 24 hours through. All earth worms in groups I—IV were put into Petri dishes of the same size filled with the same volume of spring-water, and the equal number of worms placed into each Petri dish. 100 U./50 ml SP (9 U./mg potency) were added to the Petri dishes assigned to groups III and IV. Controls for groups I and II were kept in a vessel filled with earth in the laboratory for 24 hours. Groups I and II served for comparison to groups III and IV. The temperature of surroundings was the same for all groups and controls. In-toto fixation of the worms was carried out in Bouin's fluid. Paraffin was used for embedding. Staining of the slides was made according to methods by Florentin, Gomori and Bargmann, and the azan-H method.

Results

A. PHYSIOLOGICAL OBSERVATIONS

Phenomena of locomotion and peristaltic reptation are very powerful in worms exposed to light (Group II). Considerable slowing down of movements, however, was observed in those simultaneously exposed to SP (Group IV). This group, moreover, exhibited a slower reflex contraction in response to a blow on the base (vibrations of base), and the worms in this group also had a fainter coloration than those in group II.

B. HISTOPHYSIOLOGICAL OBSERVATIONS

1. Influence of darkness (Group I)

Cerebroid ganglia. — a-Type neurosecretory cells situated in the peripheral region of the dorso-caudal part of the ganglia are hyperchromatic and contain a homogenously phloxinophilic or chrome alum-hematoxylin-(CAH)-positive cytoplasm (Fig. 1). The nuclei of these cells are spherical, flattened or irregular and rich in chromatin. In the majority of cells the nucleoli are unequal in size and positioned centrally. The cells situated in the immediate vicinity of the intercerebral part (pars intercerebralis) are larger than those previously described, and also contain much CAH-positive secretion either formed in coarse particles, or as a homogenously confluent content of the cytoplasm.

b-Type cells with basophilic cytoplasm show irregularly distributed granulations. Their nuclei are spherical or ovoidal, the nucleoli are weakly phloxinophilic.

c-Type cells are of varying sizes, irregular shape, and a wrinkled surface. The cytoplasm is slightly basophilic, the nuclei disfigured, and frequently pycnotic. This holds for cells from the peripheral, as well as for those from the middle part of the ganglia. The intercellular spaces are edematous.

Subpharyngeal ganglion. — The nerve cells responded to darkness in an identical manner as those from the cerebroid ganglia. Individual neurosecretory cells are extended in one dimension, with oblong ovoid nuclei which contain a greater amount of chromatin than the controls. The neurosecretory substance is hyperchromatic (Fig. 2A). Neuroganglionic cells corresponding to c-type cells from cerebroid ganglia are also misshaped. The cytoplasm contains unequal vacuolae and is less basophilic than in controls. The edges of nuclei often show indentations, and some of them are entirely disintegrated.

2. Influence of light (Group II)

Cerebroid ganglia. — Structural changes in response to the action of light are completely different in character from those observed in Group I. The majority of neurosecretory cells are devoid of any CAH-positive contents. In some of them there are tiny granulations, either

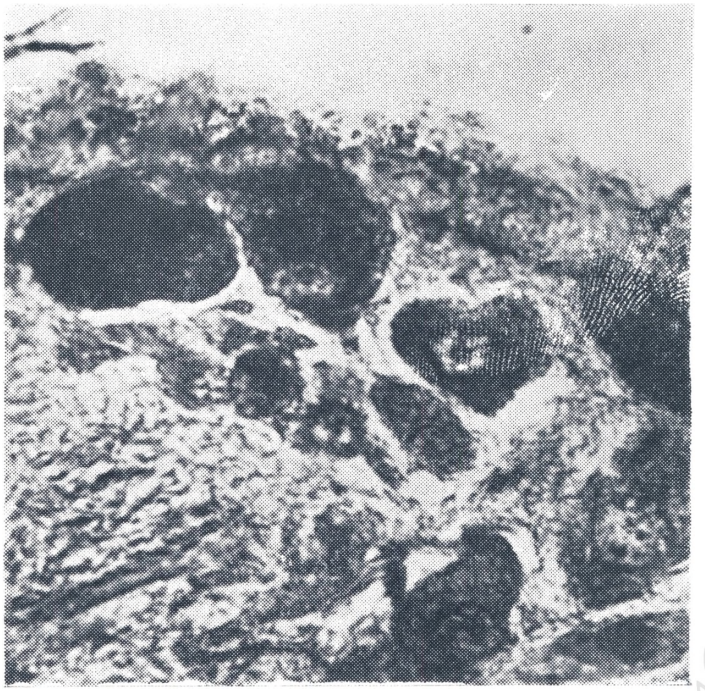


FIG. 1

Cerebroid ganglion from earth worm, Group I.
Cells filled with CAH-positive secretion (Bouin, Gomori-Bargmann, oc 8, obj. imm.)

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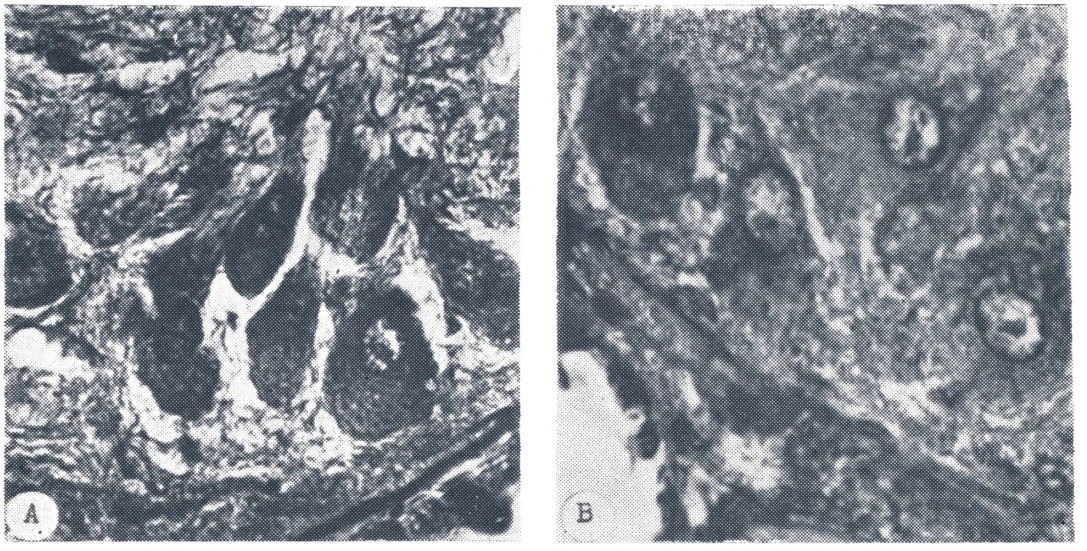


FIG. 2

Subpharyngeal ganglion from earth worm.
A, Group I, extended cells, well delimited; cytoplasm contains much CAH-positive secretion. — B, Group III, cell limitation unclear, nuclei hypertrophied, cytoplasm vacuolised; reduced amount of neurosecretion (Bouin, Gomori-Bargmann, oc. 8, obj. imm).

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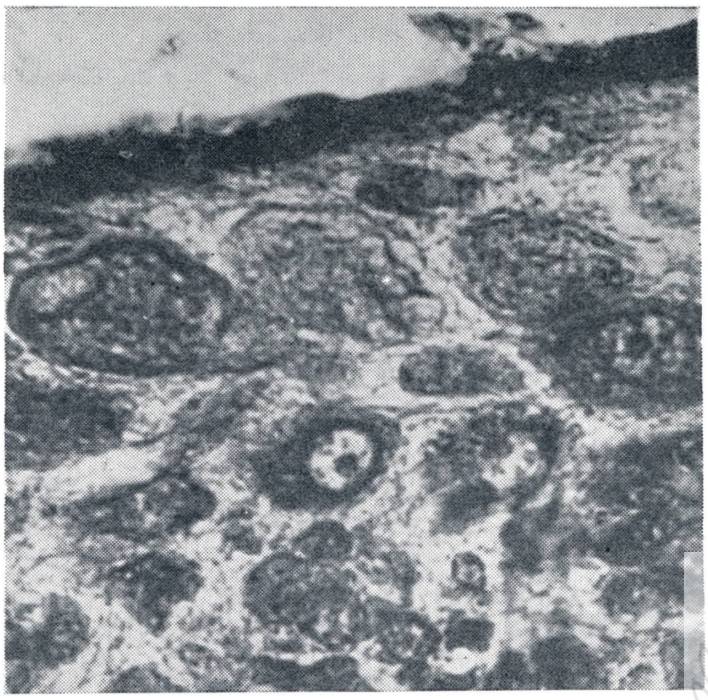


FIG. 3

Cerebroid ganglion from earth worm, Group II.
Large neuroganglionic cells with vacuolised cytoplasm (Bouin, Gomori-Bargmann, oc. 8, obj. imm.).

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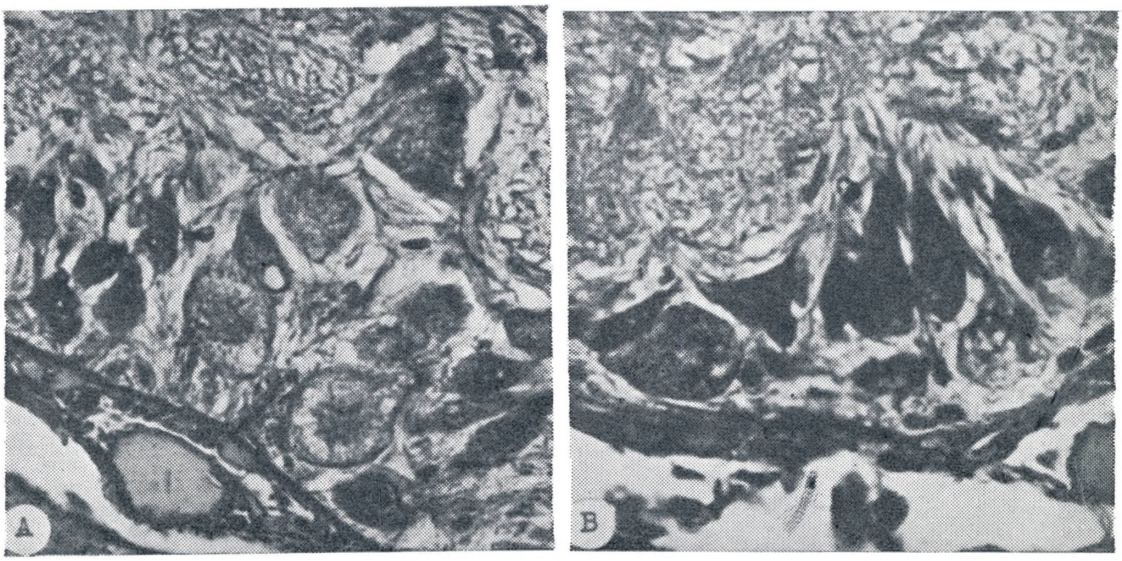


FIG. 4

Subpharyngeal ganglion from earth worm.
A, Group II, hypertrophy of neuroganglionic cells, vacuolised cytoplasm; sparse cells contain neurosecretion. — B, Group IV, shrunk neuroganglionic cells, neurosecretion blocked (Bouin, Gomori-Bargmann, oc. 8, obj. imm.).

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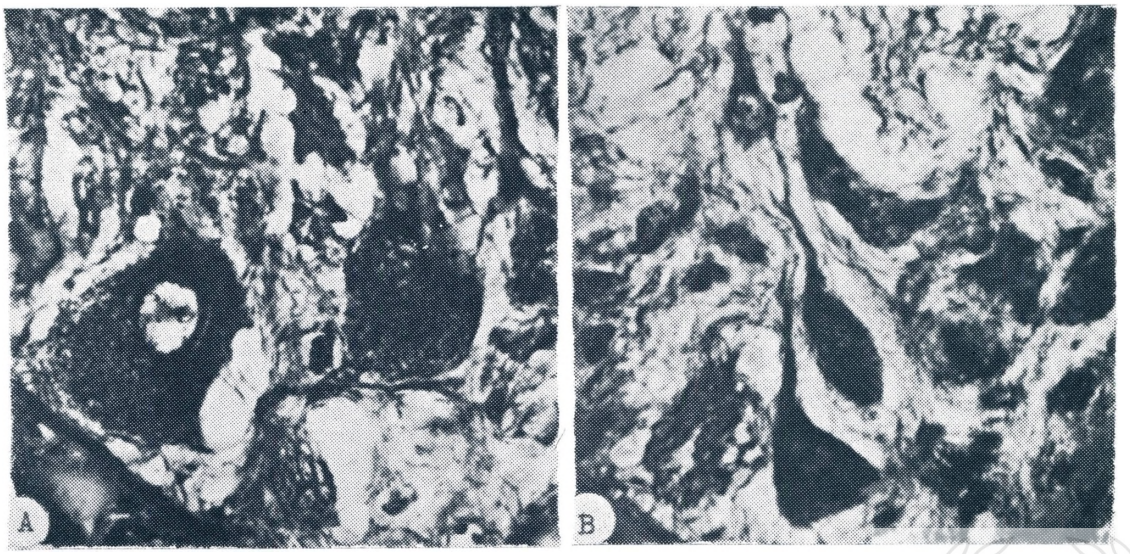


FIG. 5

Subpharyngeal ganglion from earth worm.
A, Group II, — a-type cell with peripheral vacuolae. — B, Group IV, disfigured a-tupe cells, its extensions filled with hyperchromatic neurosecretion (Bouin, Gomori-Bargmann, oc. 8, obj. imm.).

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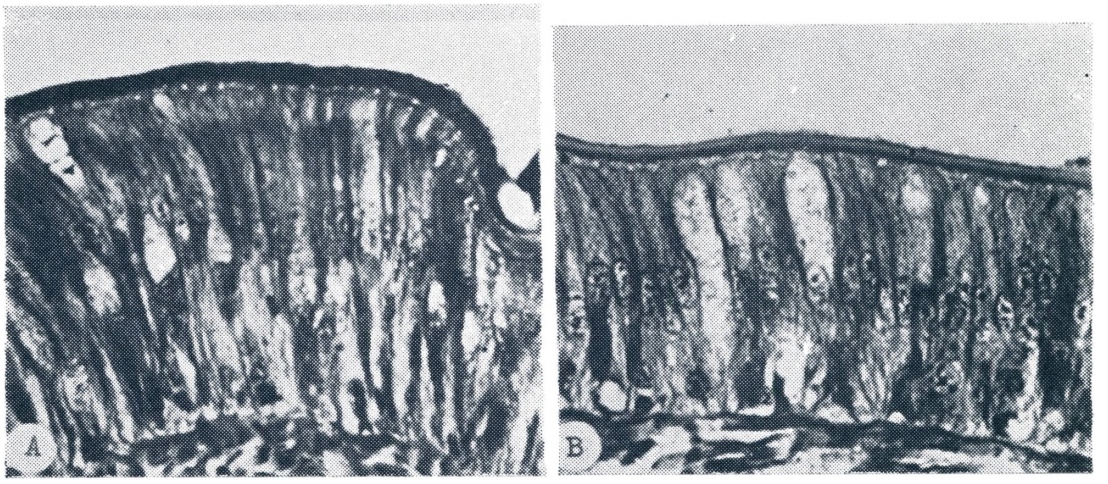


FIG. 6

Epidermis from earth worm.
A, Group II, long epithelial cells from dorsal part. — B, Group IV, epithelial cells from dorsal part shortened, cuticle thinner than in preceeding worm (Bouin, Florentin, oc, 8, obj. 60).

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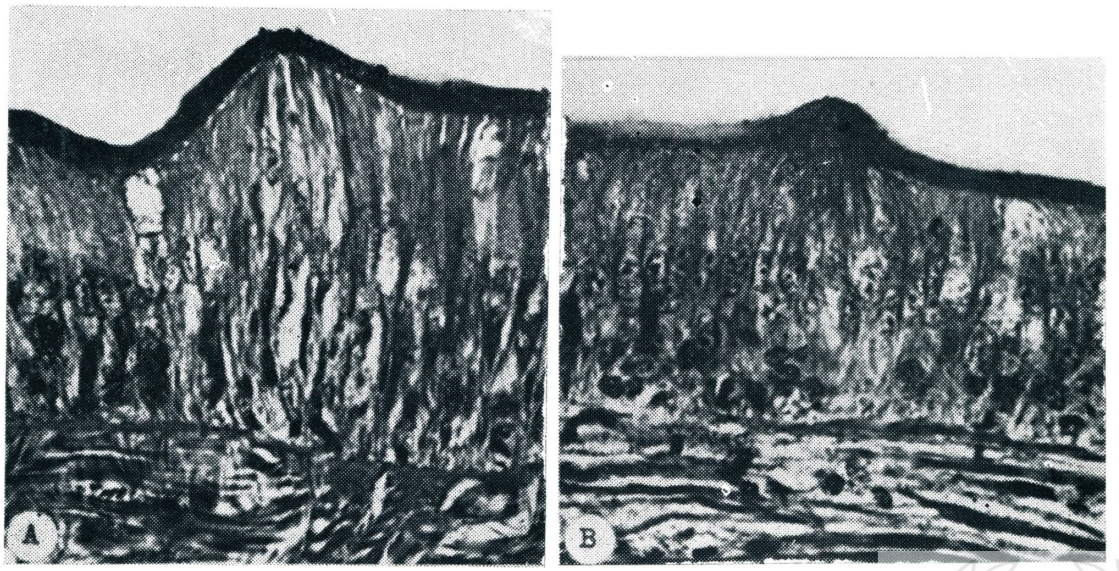


FIG. 7

Epidermis from earth worm.

A, Group II, disjoined tacto-receptor cells with atrophic, extended nuclei. — B, Group IV, compact tacto-receptor cells in a shortened epidermis (Bouin, Florentin, oc. 8, obj. 60).

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diffusely distributed, or grouped around the nuclei; among these cells those with phloxinophilic cytoplasm and hypertrophied, excentrically positioned nuclei prevail. The nucleoli are also hypertrophied, hyperphloxinophilic, and totally displaced towards the periphery, adjacent to the cell membrane. Individual cells contrast the rest by their dimensions and possess a homogenously stained or granular acidophilic cytoplasm. These cells lie close to the capillaries, which in turn show widened lumina. b-Type cells are also hypertrophic. The presence of large ganglionic cells with vacuolised cytoplasm, having a greyish tint when treated according to Gomori and Bargmann, represents one of the essential distinctions in the reactivity of cerebroid ganglia to light (Fig. 3). The nuclei of these cells, frequently carrying transcellular capillaries, are bubble-shaped, pallid, and have a very thin membrane. Around the periphery of these cells there is a chain of vacuolae larger than those from the inner part of the cytoplasm. Some of these vacuolae burst and pour out their contents into the neuropil. More numerous phloxinophilic fibres are observed than in the preceeding group. The net of capillaries is highly ramified and the lumina of capillaries dilatated.

Subpharyngeal ganglion. — Individual neuroganglionic cells are considerably larger than in controls. This is true likewise of cells with basophilic, and of extended cells with vacuolised cytoplasm. The nuclei of the latter are bubble-shaped with a hyperphloxinophilic large nucleolus. There are less CAH-positive cells than in controls.

3. Influence of darkness and SP (Group III)

Cerebroid ganglia. — The tissue pattern described in Group I is considerably modified by the presence of numerous cells without distinct limitation. Individual nuclei are larger than in Group I, excentrically positioned, bubble-shaped, with little chromatin. The nucleoli are strikingly larger than in Group I and also peripherally positioned. Neurosecretory cells are recognized by a perinuclear CAH-positive zone. Coarsely vacuolised, honneycomb-shaped cells are found in the median region near the intercerebral part. They are most intimately connected by capillaries. These cells also pour out the contents of their peripheral vacuolae into the neuropil. The net of capillaries is more marked than in Group I.

Subpharyngeal ganglion. — Cells and nuclei are larger than in Group I, with less chromatin in the nuclei. The nucleoli are also considerably larger. These changes are observed in both CAH-positive neurosecretory cells and those with basophilic, vacuolised cytoplasm. The delimitation of the latter cells is less visible.

4. Influence of light and SP (Group IV)

Cerebroid ganglia. — The general character of changes in ganglionic structure in response to light and SP is represented by smaller

dimensions of the cells in comparison to those in Group II. The nuclei, too, have a smaller volume, and show an ovoid or oblong form with irregular edges. They contain more chromatin and strikingly smaller sized, less phloxinophilic, nucleoli. Especially the vacuolised cells are fewer in number and smaller in size, but this is also the case with the cells containing acidophilic cytoplasm. The capillaries are less twisted and show a narrower lumen.

Subpharyngeal ganglion. — The structural characteristics are identical with the involutive changes described for the cerebroid ganglia. There are more cells with CAH-positive content in the cytoplasm than in Group II, and the number of vacuolised neuroganglionic cells is strikingly smaller (Figs. 4 and 5).

Discussion

The cytological characteristics of cerebroid and subpharyngeal ganglionic cells in Group I are opposite to those in Group II. They are involutive in the former, and progressive in the latter. Thus, darkness acts depressively, and light acts stimulatively not only on neuroglandular, but also on other nerve cells which, under normal circumstances do not show any neurosecretory activity. In trying an interpretation of these findings it is necessary to have in view that the experiments were carried out with earth worms in a fluid medium, whereas the controls were only kept in moist earth. To the experimental animals the staying in a fluid medium represents in itself a reactive change of surrounding conditions, which, in order to keep up the osmo-regulating balance, calls forth the response of primarily those neuroglandular cerebroid ganglionic cells playing some rôle in the osmo-regulating mechanism (Aros and Bodnar, 1960). The results obtained with the first two groups must, therefore, be interpreted as a combined effect of darkness, light, and change of surroundings.

As in Groups I and II the neuro-cytological responses in Groups III and IV, which in addition to the influence of light and darkness have been submitted to the action of SP, are also opposite. The hypertrophy of nuclei and nucleoli, the mobilisation of neurosecretory activity, the vacuolisation of the cytoplasm and the hyperemia observed in Group III reflect the stimulated activity of neuroganglionic cells; the shrinking of nuclei and nucleoli, the block of neurosecretion, and the homogenisation of cytoplasm in Group IV are indicative of certain inhibitory actions. Hence the neurotropic properties of SP are of a stimulating character in the dark, and of an inhibitory character in light. These findings point in favour of a conditioned neurotropic activity of SP, i. e. the effects of SP in one and the same nervous substrate will be different depending on the influence of various ecologic factors. Insofar as the neuroglandular cells of cerebroid and subpharyngeal ganglia are concerned, SP is a stimulant under influence of darkness, and an inhibitor under influence of light. The biological effects of SP in these

experimental conditions thus depend on the physiological state of the responding organ.

But there is a further question of importance for the interpretation of the results described, namely that about the behaviour of the SP-preparation itself. It is possible that the polypeptidic components are subject to photochemical degradation and the effects of the preparation are, in consequence, different in light and darkness. There is furthermore a possibility of other components present as impurities to act in light, but not in dark, or vice versa. Only further studies with purest SP will enable us to elucidate this side of the problem.

In discussing the present experiments one must keep in mind that they have been performed with worms reacting under a stress. Light as the stress-producing factor is especially interesting in this respect. Its nocive action on the earth worm and the sensitivity of the latter to intense light irradiation is well known (Avel, 1959). The light stimuli are transmitted through photoreceptive cells situated in the worm's epidermis to the cerebroid and subpharyngeal, as well as to ventral ganglia (Avel, l. c.). By an articulation of these first sensory or afferent neurons to the correlated efferent or motor neurons in the subpharyngeal ganglion simple reflex arcs are formed through which the mechanism of retarded contraction takes place in order to enable locomotion. Likewise, by articulation of photo-receptor neurons to efferent neurons from the cerebroid ganglia, whose axon endings enter the giant fibres (»neurocordes«), other reflex arcs are constituted, which, in contrast to the former, are intended for fast transmission in order to produce locomotion, i. e. reptation. Now light accelerates the movements of the worms, whereas SP, under the same conditions, i. e. in light, retards them as well as the morphodynamics of neuroganglionic cells described before. Thus one could raise the question about the point of attack of SP in the reflex arcs. Epithelial and glandular cells in the epidermis are the ones which show maximum shrinkage under influence of SP (Figs. 6). The CAH-positive cellular secretion accumulates most and is eliminated least from these cells. On the other hand photo-receptor cells, after exposition to light, are better conserved in earth worms treated with SP than in untreated ones. Thus SP protects epidermal photo-receptor cells from light, i. e. it protects the integrity of the sensory neuron. This effect of SP is, however, opposite to that on transmittory, i. e. the correlated motor neurons in animals exposed to light. All these histo-physiological data indicate an anti-stress action in SP. The action of SP in the adaptation syndrome to light favours the conservation of the integrity of the sensory neuron, and, at the same time, inhibits the activity of the effector neuron. This means that the distinctly sensory photo-receptor cells are receptors of SP.

In analogy to the considerations about the rôle of photo-receptor cells we also have to consider the reaction of worms to tactile stimuli (vibrations of base) in the presence or absence of SP. Untreated worms

in light react to a shock of the base by strong rapid movements. These movements are sensibly reduced in worms treated with SP. As with photo-receptor cells a protective action of SP could be observed in tacto-receptors in the epidermis. These devices include cells the structure of which, after exposition to light, is better conserved in worms treated with SP than in untreated worms (Fig. 7). Thus here again the depressive effect of SP on movements caused by sensory stimuli (light, vibration) is due to inhibition of effector, i. e. motor neurons. From all this it may be concluded that SP interferes with the transmission of sensory stimuli in the earth worm reacting under stress; its effects on sensory neurons are protective and those on the correlated effector neurons inhibitory.

The pigment particles below the epidermis, as well as in connective tissue, and especially in circular muscles are more numerous in worms exposed to light. In worms treated with SP the number of pigment particles following exposition to light is greatly reduced. Since the cumulation of pigment (porphyrin) is a response to the stress-producing effect of light in worms, and, on the other hand, the pigment metabolism is related to neurohormonal adaptive regulation, this finding is one more proof for the anti-stress activity of SP in the earth worm.

Summary

The neuroganglionic cells of cerebroid and subpharyngeal ganglia in the earth worm undergo depressive changes in darkness and progressive ones in light. SP, under the same experimental conditions, exerts the opposite effects. This may be due either to the physiological condition of the reacting organ or to metabolic changes of SP itself as a result of photochemical reactions.

In light the earth worm reacts to shock of base by intensified and accelerated movements. SP depresses this response.

From morphodynamics of afferent, sensory neurons (photo- and tacto-receptor cells in the epidermis) and the efferent, motor neurons constituting simple reflex arcs with the former, observed in the earth worm under stress, it is concluded that SP exerts an »anti-stressogenic« effect.

In light-produced stress SP protects the sensory neurons and inhibits the correlated effector neurons.

Sensory cells in the epidermis are receptors of SP.

UTJECAJ SP NA NERVNI SISTEM KIŠNE GLISTE

Neuroganglijske ćelije cerebroidnog i subfaringealnog gangliona kišne gliste pokazuju depresivne promjene u tami, a progresivne u svjetlu. Pod istim eksperimentalnim uvjetima SP izaziva suprotne efekte. To može biti posljedica bilo fizioloških uvjeta u kojima se nalazi organ koji reaguje bilo metaboličkih promjena same SP uslijed fotokemijskih reakcija.

U svjetlu kišna glista reagira na potres baze pojačanim i ubrzanim pokretima. SP smanjuje ovu reakciju.

Iz morfodinamike aferentnih, senzornih, neurona (foto-receptorske i takto-receptorske ćelije epiderme) i eferentnih, motornih, neurona koji s prvima sačinjavaju jednostavne refleksne lukove, opaženih pod stresom, zaključuje se da SP ima antistresogeni efekat.

Kod stresa prouzrokovanog djelovanjem svjetlosti, SP zaštićuje senzorne neurone i djeluje inhibitivno na korelativne efektorske neurone.

Senzorne ćelije epiderme su receptori SP.

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DISCUSSION

LEMBECK: Is SP present in the earth worm? — What are the effects of ACh, noradrenaline and 5-HT, e. g., on motility and neurosecretion? Have you compared the effects of pineal gland extracts and melatonin?

MILIN: According to the literature there have been no attempts to look for SP in the earth worm. In examining 5-HT we obtained completely opposite effects to those of SP on both motility and neurosecretion. We have made no comparison between the effects of aqueous pineal gland extracts and melatonin.

UMRATH: Do the cells in the earth worm exposed to light in presence of SP resemble those in worms kept in the dark treated with SP?

MILIN: No. The cytodynamics of the photo-receptor cells in the epidermis, as well as the glandular cells under influence of SP depends on this physiological condition: the cells react differently in light and darkness.

VOGT: Have you tried to apply hypnotics to the earth worm exposed to light?

MILIN: Such experiments have not been carried out. I want to emphasize finally that a study of the relation of SP and pineal gland deserves a particular interest. The results of preliminary examinations made in collaboration with professor Stern and his colleagues indicate the presence of SP in the pineal gland. Other results obtained about the part played by the pineal gland in the adaptation syndrome show clearly the importance of this gland in the biology of SP, and particularly in the study of stress.

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P. STERN AND S. HUKOVIĆ

SPECIFIC ANTAGONISTS OF SUBSTANCE P

Many authors interested in the SP-field have tried to find its specific antagonists. Pernow (1953) in his monography discussed antagonists of SP particulary those belonging to the class of ganglion blocking agents but none of these was specific. The existence of specific antagonists of SP has very often been inferred in the literature, but as far as we know none has really been found, nor has any enzyme specifically destroying SP yet been isolated. Enzymes which have been found to destroy SP also destroyed other polypeptides and biogenic amines. No suitable specific pharmacological test for the identification of SP has been found either, except those using animal gut, particularly the guinea pig ileum.

Krivoy (1957), in Gaddum's laboratory, found that LSD potentiates the effect of SP and Elliasson (1958) in von Euler's laboratory succeeded to show that patuline inhibited the effect of biogenic amines leaving the effect of SP unimpared. These findings could be reproduced in our laboratory and partly enable us to identify SP.

We have been screening possible antagonists of SP for more than 6 years. A great deal of drugs have been included, which seemed promising on a theoretical basis. With respect to possible interactions with SP drugs could be divided in to 3 groups. (1) Drugs which are not at all, or are only unspecific, antagonists. (2) Drugs which are specific antagonists facultatively, and (3) drugs which are really specific antagonists. Having examined a great number of substances along these lines we wish to present the results of this work.

Methods

A piece of guinea pig ileum was suspended in an isolated-organ bath in Tyrode's solution. The temperature of the solution was 32°C. Air was bubbled through the bath and the substance to be tested was added. After recording the response, another piece of gut was used for the next antagonist.

The activities of antagonists against different agonists were compared on ground of Schild's pA_2 (1947). The pA_2 is defined as the negative logarithm, to base 10, of the molar concentration of an antagonistic

drug which reduces the effect of a double dose of an active drug (agonist) to that of a single dose.

The agonist was injected every 3 min., left 30 sec. and than washed out. The contractions were recorded with a frontal lever. 3—4 contractions were allowed, first with the single dose, and than one contraction with the double dose, 2 min. before applying the double dose the antagonist was added in such a concentration that a slightly higher contraction was obtained with the double, than with single dose. The piece of ileum was than changed for a new one and, after a period of adaptation of 15 min., 3—4 control contractions were recorded again, and the antagonist was given in such a concentration, that the double dose of agonist gave a contraction of slightly less height than the control single dose contraction. The height of the double dose contraction expressed as per cent of the last single dose contraction was plotted as the ordinate against the log. of molar concentration of antagonist. The 100% value was found by interpolation and the pA_2 calculated.

Solutions were made from different stock of SP, which had varying activities: 5.3, 16.3 and 270 U./ml, and (an SP prepared in our laboratory) 3—5 U./ml. Final concentrations of the active drugs for most of our experiments were: SP 0.50 U./ml (SP 5.3 U./ml kindly supplied by N. V. Organon, Holland), ACh, 0.02 μMol/ml, 5-HT, 0.10 μMol/ml, H, 0.04 μMol/ml, $BaCl_2$, 150μMol/ml, bradykinin 0.02 μg/ml.

Results

The series of drugs shown in Table I did not exert any inhibitory effect neither on SP, nor on other polypeptides and biogenic amines, in reasonable concentrations of about 1 mg/ml. There have been, in this series, drugs with unspecific inhibitory effects on the activity of SP and still more potent effects on biogenic amines, like atropine, scopolamine, quinine, tolserol and others.

TABLE I

ANTAGONISTS OF SP
UNSUCCESSFUL AND UNSPECIFIC ANTAGONISTS OF SUBSTANCE P

Adenosine, Adermine, gama-Aminobutyric Acid, 1-AMP, 2-AMP, Aneurine, Antimit, Antipernicin, Adenosine Triphosphate, Avacan, Azulen Benzochquinone, Biotine, B O L, Buscopan, Catechol, Citric Acid, Codeine, Coffeine, Cysteine, Decholin Sodium, Desoxyribonucleinic Acid, Eutison, Dilatol, Gluthathion, Heparine, Histamine, Hydrochloric Acid, Hydrasines, Lactoflavin, L S D, Marsilid (Iproniazid), Mephenesine, Meprobamate, 1, 2-Naphthoquinone, 1, 4-Naphthoquinone, alfa-Naphthylamine, beta-Naphthylamine, N-(Naphthyl)ethylendiamine, Narceine, Nepresol, Oxytocine (Syntocine), P A B A, Patulin, Phenuron, Poliethylen Sulfate, Protamin, Quiloflex, Quinine, 8-Hydroxyquinoline, Renin, Scopolamine, Segotin, Semicarbazide, Sparteine, Strychnine, Thiosemicarbazide, Trimethadione, Urea, Vasopressin, W-181.

Another, smaller series of drugs, were facultative inhibitors like 5-adenylic acid, narcotine and acetyl aneurine which were, from time

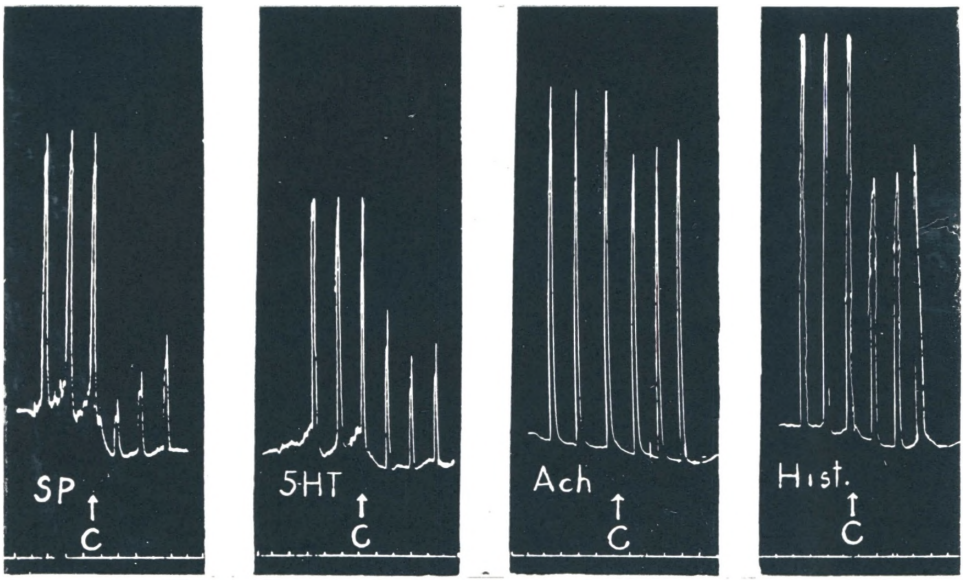


FIG. 1.

The effect of cysteine-di-β-naphtylamide (CdN) on the contractions of the isolated guinea pig ileum. Contractions are produced by active substances: SP, 0.5 U./ml; 5-HT, 10^{-6} Mol.; ACh, 2×10^{-8} Mol.; H. 6×10^{-8} Mol. The active substances are added every 3 min., left for 30 sec., then washed out. At the arrow, CdN (1.2×10^{-3}) was added and left for 2 min. The effect of active substance is then recorded, Contractions come back to the initial level slowly, after washing out.

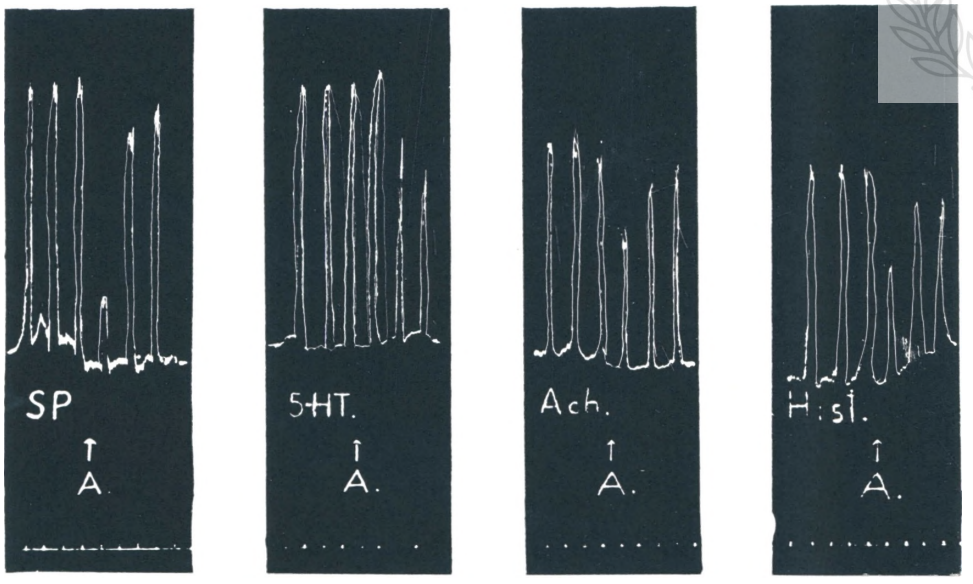


FIG. 2

The effect of Arfonad (A) on the contractions of isolated guinea pig ileum. Contractions are produced by active substances: SP, 0.5 U./ml; 5-HT, 10^{-6} Mol; ACh, 2×10^{-8} Mol; H, 6×10^{-8} Mol. The active substances are added every 3 min; left for 30 sec., then washed out. At the arrow Arfonad is added, and left for 2 min. The effect of active substance is then recorded. Contractions come back more quickly to the initial level after washing out than when CdN is used.

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to time and from animal to animal, more or less specific. pA_2 for this series is shown in Table II.

TABLE II

ANTAGONISTS OF SUBSTANCE P
pA_2 OF FACULTATIVE SPECIFIC ANTAGONISTS OF SP

Drug	SP	5-HT	ACh	H	Bradykinin	$BaCl_2$
5-Adenylic acid	5 3599	4.7510	3.7748	4.4150	4.8750	4.3400
Narcotine	4.8056	4.9420	4.1088	4.6910	—	—
Acetylaneurine	—	—	—	—	—	—

The third and most important series of drugs is that of the specific antagonists, which antagonized 4—5 times more SP than other polypeptides and biogenic amines. These drugs are: cysteine-di-β-naphtylamide and Arfonad. Hexamethonium could also be included into this group. The pA_2 for this series can be seen in the Table III.

TABLE III

ANTAGONISTS OF SUBSTANCE P
pA_2 FOR SPECIFIC ANTAGONISTS OF SUBSTANCE P

Drug	SP	5-HT	ACh	H	Bradykinin	$BaCl_2$
Cystin-di-beta--Naphthylamide	5.2499	4.7688	4.6036	4.5680	4.8170	4.0400
Arfonad	5.0300	3.9495	4.3685	4.5280	—	4.5550
Hexamethonium	3.5650	3.0000	3.0000	3.0000	—	—
2-Acet-Naphthalide	5.7450	5.5380	4.1700	4.2650	—	—

For reasons of qualitative comparison and control of the calculated values for specific antagonism the antagonistic effect of cysteine--di-β-naphtylamide is shown in Fig. 1. The effect of SP is the most inhibited of all other antagonists.

Arfonad inhibited the most SP in comparison with other active substances, like 5-HT, ACh and H as it has been shown in Fig. 2. It is interesting to note that the effect of 5-HT was potentiated after Arfonad. All active substances, after washing out of the antagonist reassume the former height of contraction.

Discussion

There are 3 groups of antagonists of SP. The first group includes unspecific antagonists, the second antagonists which are more or less specific, from animal to animal, and the third group is constituted by substances which are always the most potent inhibitors of SP as compared to other active drugs, which cause contraction of the guinea pig

ileum. The most active drugs in this series are cysteine-di-β-naphtylamide and Arfonad. The antagonistic effect of hexamethonium is not so easy to demonstrate, because great concentrations of hexamethonium alone cause the contractions of fresh ileum.

Tuppy (1959) showed that the substrate of oxytocinase is not only oxytocine but also cysteine-di-β-naphtylamide (CdN), a simple dipeptide. This relation of CdN to oxytocinase was in view of its peptide structure the reason for this investigation. It could be shown that this simple peptide inhibits SP more strongly than other active substances used in our experiment. The oligopeptide gluthathione did not possess this property. The moieties, also, of the CdN molecule, cysteine and naphtylamine are no specific inhibitors.

Pernow (1953) investigated the effect of ganglion blocking agents, like nicotine, tetraethylammonium and hexamethonium on SP activity. We have picked out Arfonad, which excedes the tetraethylammonium potency 30 times as the ganglion blocking agent to be assayed against SP. From our experiments it could be concluded that Arfonad has the same effect on SP like CdN and that Arfonad is also a specific antagonist. This substance is structuraly similar to biotine, but biotine did not show any antagonistic activity. Hexamethonium in a concentration of 0.40 to 1.0 M causes contraction of the guinea pig ileum by itself. On these contractions the contractions produced by SP did not superpose, but they do superpose on the contractions produced by biogenic amines. The stimulating effect of hexamethonium did not allow a straightforward conclusion of its exerting specific antagonism.

Laszlo (1960) proved that crude SP is accompanied, among other impurities, by 5-adenylic acid. This compound depresses the contractions of guinea pig ileum. It can be seen from our results that 5-adenylic acid reduces the effect of SP and active substances. The specificity varied from animal to animal and from time to time. We think that these variable results are caused by a different activity of adenosine monophosphatase, which destroys 5-adenylic acid. The difference probably lies in the animals, for we did not always have the same breed. The solution of 5-adenylic acid has to be made fresh, and used within a short time. Zetler (1959) noticed in his experiments that certain solutions of SP lost their protective activity, if they were left for more than 15 min. in solution.

The inhibitory potency of narcotine was the same with 5-HT and SP. This alcaloid could be used in a mixture of different antagonists, which do not inhibit 5-HT, but inhibits SP and some other biogenic amines. In this way a combination of 2 or 3 antagonists could be composed and an antagonistic effect against SP could thereby be achieved.

The most specific SP-antagonists from all drugs investigated in this experimental series are dissimilar in structure. The degree of inhibition and specifity is not so distinctive as the antagonism between atropine and ACh or LSD and 5-HT, but we think it will give us a clue to identify SP under certain conditions. These findings, also,

prompt us to try to find more specific antagonists among peptides and ganglion blocking agents.

Summary

Using Schild's pA_2 scale a series of substances were investigated as potential antagonists of SP. Three groups of substances could be combined. The first group is constituted of substances unable to produce any depression in reasonable concentrations. The drugs which are unspecific antagonists of SP are listed in the same group.

The second group comprises the drugs, which are more or less specific, from animal to animal. To this group belong: 5-adenylic acid, narcotine and acetylaneurine.

The third and the most important group are the specific antagonists. The most thoroughly investigated ones are cysteine-di-β-naphtylamine and Arfonad. These structuraly different substances have a quantitatively similar effect.

Specific antagonism is not so strong as that usually seen with biogenic amines and their specific antagonists, but it gives us some possibility to use the respective compounds as tools in further investigation, which should be turned towards peptides and very potent ganglion blocking compounds.

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SPECIFIČKI ANTAGONISTI SUPSTANCIJE P

Istraživan je niz supstancija kao potencijalnih antagonista SP. Za ova istraživanja je upotrijebljena Schildova pA_2 metoda. Nađene su tri grupe supstancija. Prva grupa su supstancije koje ne izazivaju nikakvu inhibiciju u prilično velikim koncentracijama. U istu grupu su ubrojani nespecifični inhibitori aktiviteta SP. Druga grupa su supstancije, koje su više ili manje specifične na raznim životinjama i u raznim vremenskim periodima. U ovu grupu spadaju: 5-adenilna kiselina, narkotin i acetil-aneurin.

Treća i najvažnija grupa su specifični antagonisti. Najviše su ispitivani cistein-di-β-naftilamid i arfonad. Ove dvije strukturalno različite supstancije izazivaju kvalitativno slične efekte.

Specifični antagonizam prema SP nije tako uočljiv kao kod ispitivanja biogenih amina i njihovih specifičnih antagonista, ali nam naši rezultati daju mogućnost da upotrijebimo pomenute nove antagoniste SP kao sredstva za dalja istraživanja. Traženje novih antagonista SP treba da se orijentira prema peptidima i vrlo jakim blokatorima ganglija.

ACKNOWLEDGMENT. — Thanks are due to the Federal Council for Scientific Research (Yugoslavia) for financial help.

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DISCUSSION

GADDUM: I congratulate the authors on these results. Have you tried Tubocurarine?

HUKOVIĆ: We are going to try. Dr. Varagić tried Tubocurarine and found that it inhibits SP.

STERN: It would be very important to put new synthetic peptides (oligo and poly), as well as ganglioplegic agents to test as potential antagonist of SP. Possibly much stronger and more specific antagonisms will be detected in this manner.

LEMBECK: I have tried Dibenamine and found that it is not an antagonist of SP.

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G. ZETLER

TWO HITHERTO UNKNOWN BIOLOGICALLY ACTIVE POLYPEPTIDES IN A SUBSTANCE P PREPARATION MADE FROM CATTLE BRAIN*

In 1956 and again in 1959, I described central actions of SP. When I performed these experiments I took for granted that the crude SP preparation made from cattle brain according to von Euler (1942) contains only one biologically active polypeptide, namely SP. From the results of these experiments I drew the conclusion that SP causes sedation, enhances the hexobarbital narcosis and the bulbocapnine catalepsia, diminishes chemically induced convulsions and morphine analgesia, and gives rise to a state of hyperalgesia. These findings have generally been confirmed by other authors (reviewed by Zetler, 1960) who also worked with very impure SP preparations. Recently, however, contradictory results were achieved with purified SP or with several fractions of an impure preparation:

Partially purified SP containing 100—270 U./mg, according to Stern and Huković (1960), is devoid of strychnine-antagonistic and hexobarbital-synergistic activity but still antagonizes the morphine-analgesia. Bonta, Wijmenga and Hohensee (1961) found that some fractions of an impure SP preparation have strychnine-antagonistic but no hexobarbital-synergistic or morphine-antagonistic activity. Furthermore, they got a fraction which was active against strychnine, but did not cause the isolated guinea pig ileum to contract.

These discrepancies which seem to hint at the existence of more than one centrally active component in impure SP preparations could, in part, stem from differences in the pharmacological testing methods used by different authors. This applies in particular to the methods which are supposed to determine quantitatively analgesia in mice. It is very well possible that the heat stimulus applied by Bonta, Wijmenga and Hohensee (1961) to the paws of mice according to Herr and Perszasz (1950) does not give the same information as the related method of Woolfe and Macdonald (1944) used by Stern and Huković (1960) or even

*) Detailed report: Naunyn-Schmiedeberg's Arch. exp. Path. u. Pharmak. in press (1961).

the electrical stimulation of the mouse's tail. In the latter procedure — applied by myself — the limiting criterion is the squeaking of the mouse and not a reflectory movement as in the former two methods.

It is, however, probably not justified to attach too great importance to those differences in methods, whilst one must doubt that in each case exactly the same SP material was used. In fact, up to now only very impure SP preparations were examined with respect to central actions. The SP preparation used by Stern and Huković (1960) with 270 U./mg can also be considered to be only partially purified since Pernow (1953) achieved 3,000 U./mg and Franz, Boissonnas and Stürmer (1961) even 30,000—35,000 U./mg. However, there can be no doubt that very impure SP preparations with 10 and less U./mg can exert central effects when given in doses of 5—10 U./g body weight subcutaneously in mice. Therefore, the composition of impure SP preparations deserves our interest. This applies expecially to the case that pure or synthetic SP should turn out to be completely devoid of central activity.

The chromatographic fractionation of SP made from brain or spinal cord gave in Prof. von Euler's laboratory several times only one biologically active polypeptide, namely SP. My own results are incompatible with this finding, but could have some bearing on the pharmacological discrepancies I mentioned a few minutes ago.

Results

The starting-material in my experiments was a crude SP preparation made from cattle brain according to von Euler (1942). It was the end-product of the second ammonium sulphate precipitation and, therefore, contained inorganic salts. In order to remove some of the inorganic impurities I applied to the SP powder the desalting procedure of Behrens and Seydl (1951) which means that the finely ground powder was suspended, in the dry state, in carbon tetrachloride (CCl_4). In this water-free system inorganic salts sink to the bottom of the tube whereas polypeptides go up and can be removed from the surface of the CCl_4.

I have compared the CCl_4-treated SP preparation with the untreated one and found by means of paper chromatography (solvent system *n*-butanol : acetic acid : water 40 : 10 : 50, direction ascending) for the normal SP preparation only one zone of biological activity as it could be expected. The CCl_4-treated material also had this zone, but there was, in addition, a second one which migrated much faster. A similar result was achieved by means of paper electrophoresis (three hours at pH 4.95, acetate buffer). Normally, one biologically active zone can be found on the cathodic side of the paper which agrees to the finding of Pernow and Rocha e Silva (1955). The CCl_4-treated material, however, showed besides the cathodically migrating activity a second zone, which travelled to the anode. It was, furthermore, evident that in the case of the CCl_4-treated material the cathodically migrating component comprised clearly more biological activity than one could

expect from the result achieved with normal SP. In addition to this, the form of this zone suggested that there a second cathodically migrating component could be hidden. I have checked this possibility by doubling the duration of the electrophoresis from 3 to 6 hours which split the large zone into two, which were clearly separated from each other. Thus, one reaches the conclusion that the CCl_4-treated SP preparation contains, in contrast to the untreated material, not one but three biologically active components, two of them migrating to the cathode and one to the anode.

This conclusion was corroborated by the results of column chromatography on anionotropic aluminium oxide. The aluminium oxide powder was suspended in 70% methanol and the material to be chromatographed was dissolved in the same fluid. As to the CCl_4-treated SP preparation, a first biologically active fraction (»Fa«) came out from the column whilst the starting solvent — 70% methanol — was still passing. Obviously, this first fraction was not adsorbed by the aluminium oxide. The methanol solution was followed by distilled water which delivered a second active fraction (»Fb«). This is very probably SP which, according to the experience gained in von Euler's laboratory, could be expected to leave the column at this phase of the experiment. Finally, 0.1 N sodium hydroxide solution eluted a third fraction (»Fc«). The SP preparation which was not in contact with CCl_4 yielded a great amount of biological activity (Fb) when distilled water was used as solvent. The quantities of Fa and Fc eluted with 70% methanol and 0.1 N NaOH, respectively, were considerably smaller than those which appeared after the crude SP preparation was treated with CCl_4, but there can be no doubt that the normal SP preparation used in this experiment did also contain these two biologically active components.

I have examined in some detail these three active principles by means of ascending paper chromatography using three solvent systems. System I was *n*-butanol : acetic acid : water 40 : 10 : 50, System II was *n*-butanol : pyridine : acetic acid : water 30 : 20 : 24 : 6, and System III was pyridine : acetic acid : water 35 : 50 : 15. The results are given in Table I which summarizes the more essential findings. Fa, which is the first fraction leaving the column during the passage of 70% methanol, moved in solvent I definitely slower than Fb, which left the column with water. Fc — the last fraction — moved even faster than Fb. The addition of pyridine to the solvent system alters the R_f values. Fa and Fb now travelled faster, and this increase in speed is greater for Fa than for Fb. Fc, however, now moved slower than before, and thus contrasted clearly to Fa and Fb. In solvent III, which does not contain butanol any more, all 3 fractions moved very fast but Fa was now in front of Fb and Fc.

I think that the results achieved with the aluminium oxide column and by means of paper-chromatography permit the tentative conclusion that these three biologically active principles are in fact 3 different substances.

TABLE I

SHORT SUMMARY OF THE CHARACTERISTICS OF Fa, Fb AND Fc AS ACHIEVED BY ALUMINIUM OXIDE CHROMATOGRAPHY OF THE CCl_4-TREATED SP-POWDER. COMPARISON WITH A NORMAL SP-PREPARATION AND WITH SYNTHETIC BRADYKININ

	Rf-values (ascending paper chromagraphy)			Migration during paper electrophoresis (pH 4.95, acetate buffer, duration 6 hrs)	Enzymatic destruction	Action of isolated guts*)	Action on isolated rat's duodenum	Hypotensive action**) (atropinized rabbit)	Contracting action**) on isolated rat's uterus
	System I	System II	System III						
Fa	0.22	0.41	0.81	107 mm to cathode	by trypsin, chymotrypsin and pepsin	fast	?	1	2
Fb	0.34	0.46	0.61	57 mm to cathode	by trypsin, chymotrypsin and pepsin	fast	contracting	1	1
Fc	0.64	0.54	0.75	80 mm to anode	*not* by trypsin, but by chymotrypsin, pepsin and papain	slow	contracting	0.025	0.2
normal SP-preparation	0.35	0.43	0.66	116 mm to cathode	by trypsin, chymotrypsin and pepsin	fast	contracting	1	1
synthetic Bradykinin	0.32	0.4	0.76	50 mm to cathode	*not* by trypsin, but by chymotrypsin	slow	relaxing	not tested	$\geqq 30$***)

*) Guinea pig's ileum and rabbit's jejunum.
**) Relative strength (SP = 1)
***) According to Gomes (1955).

This view seems to be supported by the behaviour of these 3 components during paper electrophoresis at pH 4.95. Fb — this is fraction Nr. 2 and can be considered to be SP — migrated fastest to the cathode. Its speed was the same as that of the biological activity present in a normal crude SP preparation. Fa, the first fraction from the column, travelled half as fast to the cathode and Fc — the third fraction — migrated to the anode.

Experiments in which proteolytic enzymes were applied have revealed that the principles we are dealing with are very probably polypeptides: Chymotrypsin, trypsin and pepsin destroyed the biological activity of Fa and Fb, the latter being SP. Fc, however, resisted trypsin but was destroyed by chymotrypsin, pepsin and papain. The resistance of Fc against trypsin reminds of bradykinin. There is another property which Fc shares with bradykinin: on the isolated guinea pig ileum and rabbit jejunum, Fc elicited, in contrast to Fa and Fb, a very slow, bradykinin-like contraction. In spite of these similiarities, Fc is not bradykinin, for it travelled twice as fast as synthetic bradykinin during paper chromatography with butanol : acetic acid : water, and it migrated to the anode, but bradykinin migrated to the cathode. Furthermore, it is well known that bradykinin, other kinins, oxytocin and vasopressin cause the isolated rat's duodenum to relax (Horton, 1959), but Fc elicited, in contrast to this fact, a contraction. Fb, too, caused this isolated organ to contract, which supports the view that Fb is SP. The action of fraction Fa was relaxing, due to an impurity which is neither a polypeptide, nor AMP or a related compound. It may be added that on the isolated rat uterus which is known to be about 30 times more sensitive for bradykinin than for SP, Fc is about 10 times less active than Fa and Fb, Fa being a little more active than Fb.

Fa and Fb were hypotensive in rabbits and this activity was abolished by chymotrypsin. Fc was practically inactive in this respect since 40 U./kg of Fc were less active than 1 U./kg of Fb. The very small effect of 40 U./kg of Fc was not present any more after the material was incubated with chymotrypsin.

Discussion

The data presented to you may be interpreted in the following way: Fraction Fb — the second one coming out from the aluminium oxide column — is SP, whereas Fa and Fc are two hitherto unknown biologically active polypeptides. Fc is a trypsin-resistant slow contracting polypeptide and so far, to my knowledge, the only gut contracting polypeptide which, at a pH below 7, migrates to the anode and is, therefore, acidic by nature.

The amount of these two new polypeptides in crude SP made from cattle brain is normally very low — about 20% of the total activity — but it is considerably augmented — to about 60% — by CCl_4. Since CCl_4 has a strong protein denaturating activity, these two polypeptides in CCl_4-treated material may be considered to be

artefacts arising from protein denaturing processes, which release the active polypeptides from inactive precursors. · The small amounts of these polypeptides in SP preparations which were not in contact with CCl_4 may also stem from protein denaturation, unavoidably happening in course of the preparation according to von Euler. One step which leads definitely to considerable protein denaturation is the precipitation of impurities by ethanol. The precipitated material is insoluble in water although it was soluble before and, therefore, must consist of denatured proteins.

Nevertheless, it cannot be excluded yet, that these two new polypeptides are normal constituents of brain tissue, as is SP itself.

Be that as it may, there is the possibility that these two new polypeptides did in part contribute to the central actions of crude SP preparations described by several other authors and by myself. This applies certainly to my own papers of 1956 and 1959, since in these experiments I used SP preparations which were desalted by means of the CCl_4 method. It is true, I made sure that the observed effects were linked to the polypeptide nature of the injected material, but I used a crude trypsin preparation which is known to contain chymotrypsin besides trypsin. Therefore, in these incubation experiments probably all three polypeptides Fa, Fb and Fc were destroyed and it may well be that each of them is centrally active and thus contributes to the net result achieved with the entire mixture.

Considering this, it is astonishing that other authors, working with crude SP preparations, got on principle the same central effects as myself, although their preparations were not treated with CCl_4 and, therefore, contained, according to my present experiments, probably not more than about 20% of these two new polypeptides in contrast to my own preparation with about 60%.

Now, two conclusions are possible: (1) The new polypeptides are not centrally active and, therefore, their concentration in different crude SP preparations did not modify the central effects seen by other authors. (2) The new polypeptides are centrally active but their different concentration in different crude SP preparations did not modify the pharmacological net result since additional amounts of them were, after injection, liberated from the injected inactive precursors by an »in vivo« process similar to that induced »in vitro« by CCl_4.

Only the second assumption, namely that my crude SP preparation contained at least two or even three centrally active polypeptides, can help to discuss the discrepancies I mentioned in the introduction. These discrepancies appeared so far only when purified or fractionated SP preparations were used. It could well be that during purification or fractionation the one or the other centrally active polypeptide was either released, or destroyed, or concentrated, so that the end product in question was not simply the starting material in a purer form, but perhaps something qualitatively quite different. This possibility applies

especially to cases in which during purification, organic solvents were used.

Finally, my results seem to have some bearing on the old question what happens during protein denaturation. A release of biologically active polypeptides by protein denaturation could perhaps also happen in the course of pathological events.

Summary

Small amounts of two hitherto unknown pharmacologically active polypeptides are present in crude SP made from cattle brain. The three active components of impure SP are easily separable from each other by means of column chromatography, paper chromatography and paper electrophoresis. Pretreatment of the crude powder by carbon tetrachloride greatly increases the amounts of the two new polypeptides which, therefore, can perhaps be considered to be artefacts due to protein denaturation.

DVA DO SADA NEPOZNATA BIOLOŠKI AKTIVNA POLIPEPTIDA U JEDNOM PREPARATU SP IZ MOZGA GOVEDA

U sirovim preparatima SP iz mozga goveda nalaze se male količine dvaju do sada nepoznatih, farmakološki aktivnih polipeptida. Tri aktivne komponente nečistih preparata mogu se lako separirati kromatografijom na koloni, papiru i elektroforezom. Poslije prethodne obrade sirovog praška ugljentetrakloridom, znatno su povećane količine dvaju novih polipeptida, koji se zbog toga, možda, mogu smatrati artefaktima nastalim uslijed denaturacije proteina.

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DISCUSSION

GADDUM: I congratulate professor Zetler most warmly on these results. I suggest that some of these substances should be tested on gold fish intestine particularly the substances which travelled towards the anode.

KRIVOY: It is unlikely that SP contains bradykinin since chlorpromazine potentiates the action of bradykinin but not SP in blood pressure.

ZETLER: This is in agreement with my own ideas.

PERNOW: I agree with Dr. Zetler that it is unlikely that either Fa or Fc is bradykinin. Rocha e Silva and I mixed bradykinin and SP and found it impossible to separate them on aluminium oxide by elution with methanol. It is further unlikely that bradykinin, such a crude preparation, might be eluted by methanol at such a high concentration as 70%. It is also unlikely that Fc is bradykinin since at the pH used in the buffer bradykinin should migrate to the cathode.

We usually use aluminium oxide column technique in order to both purify the substance and check the specifity of the extracts. I think that Dr. Zetler's results give further support to the opinion that the alumina chromatography technique should be put on the list of methods discussed yesterday which should be used in order to test the specificity of the SP action.

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F. LEMBECK

ASPECTS CONCERNING THE OCCURRENCE AND DISTRIBUTION OF SUBSTANCE P IN THE BRAIN

With regard to the occurrence of SP in the brain the following questions are of interest:

(a) Which cells of the brain contain SP?

(b) Which part of the cell contains SP?

(c) At what time of the embryonic development does SP already occur in the brain or in the intestine?

(a) Since about 90 per cent of the cerebral tissue consists of glia, it is impossible to say whether a substance found in a brain extract originates from nervous or glia cells. We tried to approach this question by determining the SP activity in brain tumors (Grabner and Lembeck, 1960). These tumors either consist of entirely immature, that is, undifferentiated nervous tissue or they develop from the glia at any stage of its development; the critical point, however, is that they never arise from mature nerve cells. A total of 20 brain tumors of different origin were extracted, but none of the extracts contained measurable amounts of SP when tested on the isolated guinea pig ileum under atropine and neoantergan. Two of the extracts were active in producing slow and highly atypical contractions which were not regarded as being due to SP. Since our material included samples from all the classes of the commonly occuring brain tumors we would like to conclude from these results that SP does not occur in the glia and, therefore, must come from nervous tissue. There is, however, one possibility to restrict our conclusion: all tumor tissue is, to a variable degree, immature and possibly could not have the same synthetic power to produce all the substances that occur in normal tissue. Some additional support for the location of SP in nervous elements is derived from the finding that the retina which is relatively poor in glia cells contains high amounts of SP (Dunér, Euler and Pernow, 1954). Further, there is an increase of SP in the proximal, and a decrease in the distal part after some days in a severed nerve (Andrews and Holton, 1958).

(b) Differential centrifugation of brain homogenates showed that the highest amount of SP is located in the »mitochondrial fraction« (Lembeck and Holasek, 1960; Zetler, 1961). This observation has been

extended by Gaddum (1961), who separated the mitochondrial fraction over sucrose layers and found the highest amount of SP in a granular fraction different from the mitochondria.

(c) The occurrence of SP during embryonic development was studied in collaboration with Dr. Petschke. We found, that in the brain stem of bovine fetus, SP occurs already at an early stage of development. In a brain weighing only 1.1 g which was therefore not separated into brain stem and fore brain already 21.5 U./g SP have been found. The brain stem at any time of the development already contained amounts of 21.5—65.6 U./g which are comparable to those found in the developed bovine brain. On the other hand, the fore brain was almost free of measurable amounts of SP during the whole period of embryonic development. The gut contained increasing amounts of SP during the second half of the embryonic development. It is tempting to correlate these results with respect to function: the brain stem gains its function quite early in the fetal life and is active during the development of the embryo. Concomitantly SP occurs early in that part of the brain. The cortex, on the other hand, which remains immature and quiet up to birth and some time thereafter is practically free of SP during this period. This correlation points toward the possibility of a physiological function of SP for the activity of the central nervous system.

Summary

Investigations on brain tumors indicate that SP is located in nervous elements and not in the glia.

The intracellular localisation of SP is highest in a granular fraction of brain homogenates.

During embryonic development SP was found already very early in the brain stem, in the cortex however not before birth.

POGLEDI NA PRISUTNOST I DISTRIBUCIJU SP U MOZGU

Istraživanja u vezi s moždanim tumorima pokazuju da se SP nalazi u nervnim elementima, a ne u gliji.

Intracelularna lokalizacija SP je najveća u granularnoj frakciji moždanih homogenata.

U toku embrionalnog razvoja SP se već vrlo rano nalazi u moždanom deblu; u kori se, međutim, ne pojavljuje prije poroda.

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J. FRANZ, R. A. BOISSONNAS AND E. STÜRMER

PHARMACOLOGICAL PROPERTIES OF SUBSTANCE P ISOLATED IN AN APPARENTLY PURE STATE

SP was discovered 30 years ago by von Euler and Gaddum (1931). It is a potent biogenic polypeptide and appears to play an important rôle under physiological conditions (Schachter, 1960). The krown pharmacological properties of SP reported in the literature and at this symposium are based mainly on observations with crude SP preparations. Pernow (1953) by chromatography on aluminium oxide columns succeeded in preparing a strongly enriched SP preparation with an activity of 2,500—3,500 U./mg. Recently Franz, Boissonnas and Stürmer (1961) were able to isolate SP from horse intestine in an apparently pure state (activity 30—35,000 U.*)/mg).

This apparently pure substance P (SPp) was compared with a crude substance P extract (SPex: our starting material) on isolated smooth muscle preparations (guinea pig ileum and hen caecum) and on the blood pressure of the atropinized rabbit. The results are presented in Figs. 1—3.

It can be seen from the Figures that SPp was active in all these tests and that, within the limits of biological variation, the increase in activity in all three tests was proportional to the degree of purification, viz. about 1 : 2000 (SPex : SPp). These SP activities thus appear to be inherent properties of our highly purified material which is probably mainly responsible for the pharmacological activity of crude SP-extracts.

SP behaves differently from pure synthetic bradykinin on hen caecum: as is shown in Fig. 4, SP contracts hen caecum, while bradykinin causes relaxation. On treatment with chymotrypsin (0.05 mg SPp + 0.002 mg chymotrypsin in 0.012 ml, pH 9.5, 3 hours, 25°C) SPp and bradykinin are broken down, but only the former yields arginine. Under the same conditions, trypsin does not completely inactivate SPp; carboxypeptidases (A + B) which inactivate bradykinin, splitting off arginine, do not destroy the biological activity of SPp, nor is arginine split off. Total hydrolysis of SPp and bradykinin yields arginine and

*) We are indebted to Dr. Pernow who kindly supplied us with von Euler-Gaddum standard.

proline as common constituents, but SPp also yields leucine/isoleucine and alanine, amino-acids which are not found on hydrolysis of bradykinin.

Summary

Substance P was isolated in an apparently pure state from horse intestine by chromatography and electrophoresis (activity 30,000—35,000 units per mg). The activity of the purified substance (SPp) and the starting material (SPex) were compared on guinea pig ileum, hen caecum, and rabbit blood pressure. The ratio of activities, SPp:SPex, was 2000:1.

SPp and Bradykinin were readily distinguished in their pharmacological and chemical properties.

FARMAKOLOŠKA SVOJSTVA SP IZOLOVANE U OČITO ČISTOM STANJU

SP izolovana je u očito čistom stanju iz crijeva konja primjenom kromatografije i elektroforeze (aktivnost 30,000—35,000 jed./mg). Aktivnost prečišćene supstancije (SPp) i ishodnog materijala (SPex) uspoređene su na ileumu zamorca, caecumu kokoši i krvnom pritisku kunića. Aktivnosti stoje u odnosu SPp : SPex = 2000 : 1.

SPp i bradikinin se jasno razlikuju po svojim farmakološkim i kemijskim svojstvima.

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DISCUSSION

LEMBECK: (1) Were the steps of purification carried out under low temperature?

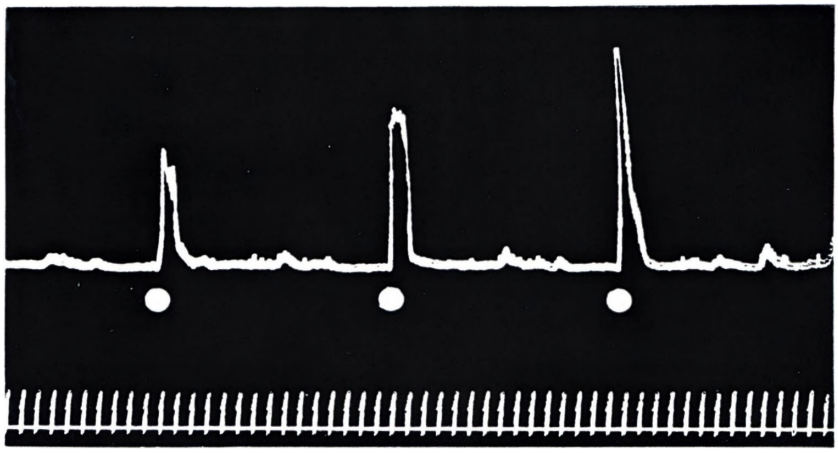
(2) In which steps of purification did the greatest loss occur?

STÜRMER: (1) The chromatography on aluminium oxide was carried out at low temperature.

(2) The greatest loss occurred in the ion exchange chromatography.

PERNOW: I want to ask you more about your experience of the stability of the pure preparation. When I worked on the purification of the substance and got a preparation which seems to have had one tenth of the activity of your most active fraction, I found it very unstable in a more purified stage. I tried to find out if this lability was due to oxidation or enzymatic activity, but I never found any consequence in the »spontaneous« loss of activity. Very often I found that if a higly purified preparation in aqueous solution was frozen down and then again thawed up a 50 per cent loss of activity or more was found.

STÜRMER: Some solutions of our highly purified SP retained their potency for about 4 days. However, sometimes a loss of biological activity was detectable within 24 hours. We do not know the reason for this.



atropine and thenalidine 10^{-6} g/ml added 5 min. before SP.

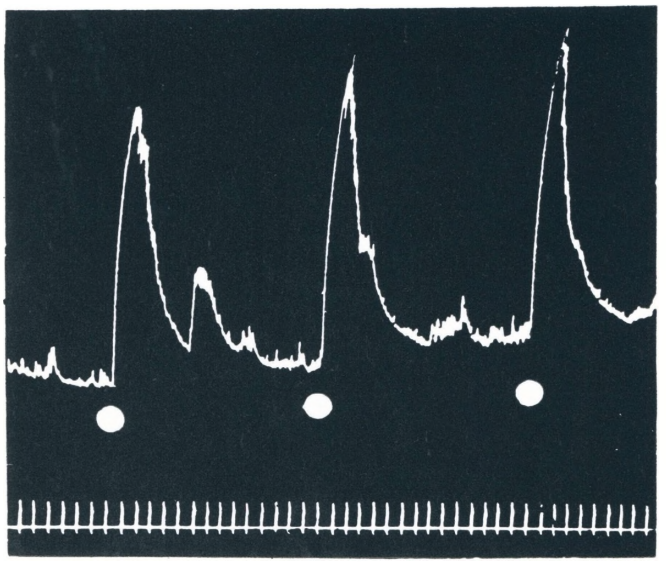
min.

0.3 U SPex	0.01 µg SPp	0.3 U/ml SPex
0.1 µg SPp	—	0.3 U
1 mg SPp	—	30,000 U

FIG. 1

Effects of a crude extract, SPex, (15 U./mg) in comparison with the highly purified SP-preparation (SPp) added (o) to isolated guinea pig ileum. Note that there is no difference between the action of crude and highly purified SP. Other details of method: Tyrode solution; 37° C; carbogen; all doses per ml; concentrations of atropine and thenalidine refer to the salts (sulphate and tartrate respectively).

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atropine and thenalidine 10^{-6} g/ml added 5 min. before SP.

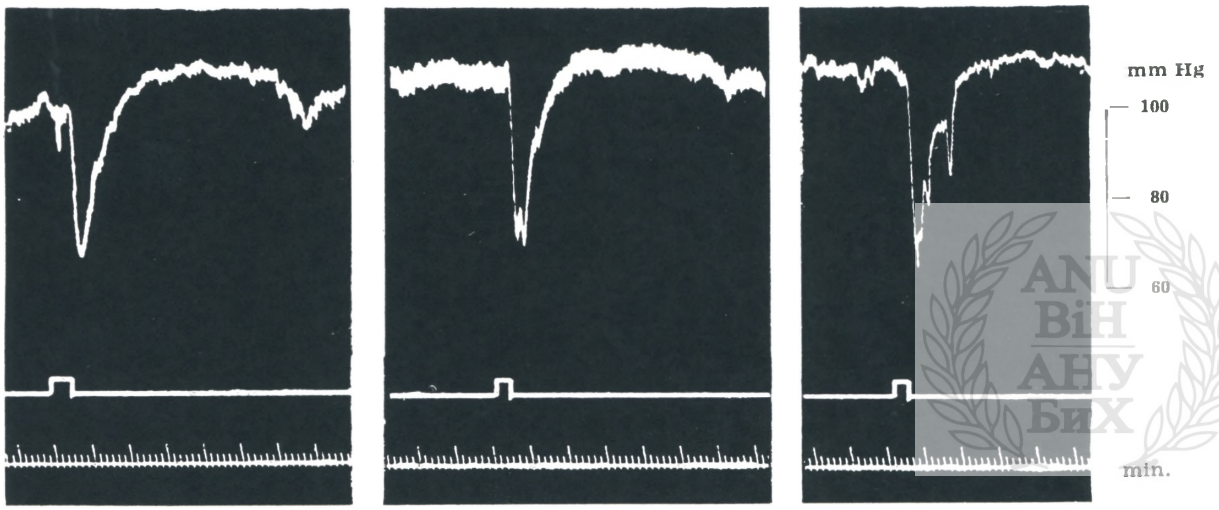
min.

0.3 U. SPex	0.1 µg SPp	0.3 U./ml SPex
0.01 µg	—	0.3 U.
1 mg SPp	—	30,000 U.

FIG. 2

Effects of a crude extract, SPex, 15 U./mg) in comparison with the highly purified SP-preparation (SPp) added (o) to isolated hen caecum. Note that there is no difference between the action of crude and highly purified SP. Method as described in Fig. 1.

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2.8 U. SPex	0.084 µg SPp	2.8 U./kg SPex
0.084 µg SPp	—	2.8 U.
1 mg SPp	—	33,000 U.

FIG. 3

Effects on intravenous injection (⨍) of a crude extract, (15 U./mg) in comparison with that of the highly purified SP-preparation (SPp) on the blood pressure of the rabbit recorded by a mercury manometer from the carotid artery.

Note that there is no difference between the action of crude and highly purified SP.

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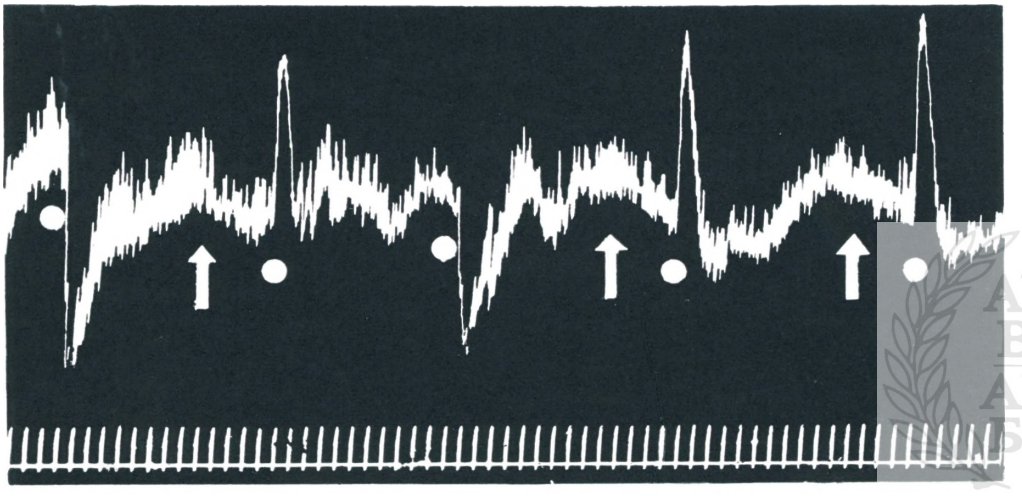


FIG. 4

Effects of SP and pure synthetic bradykinin (Br) added (o) to isolated hen caecum. Method as described in Fig. 1.

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HAEFELY: We found our purified SP preparation rather unstable when diluted in accordance with Pernow. It is surely not due to bacterial contamination and probably not to oxidation. Because of the lability of more purified SP we still prefer an impure preparation as standard. The purified SP was prepared by Hoffmann la Roche by counter current distribution. The molecule does not contain histidin.

PERNOW: Professor Gaddum mentioned the silicone treatment of the glasses in order to prevent losses of activity. I tried this once after having read in the Biochemical Journal a few years ago, that this technique prevented loss of activity of angiotensin, which was found to be easily adsorbed on the glass wall. In my experience this treatment, however, did not prevent the loss of activity of SP.

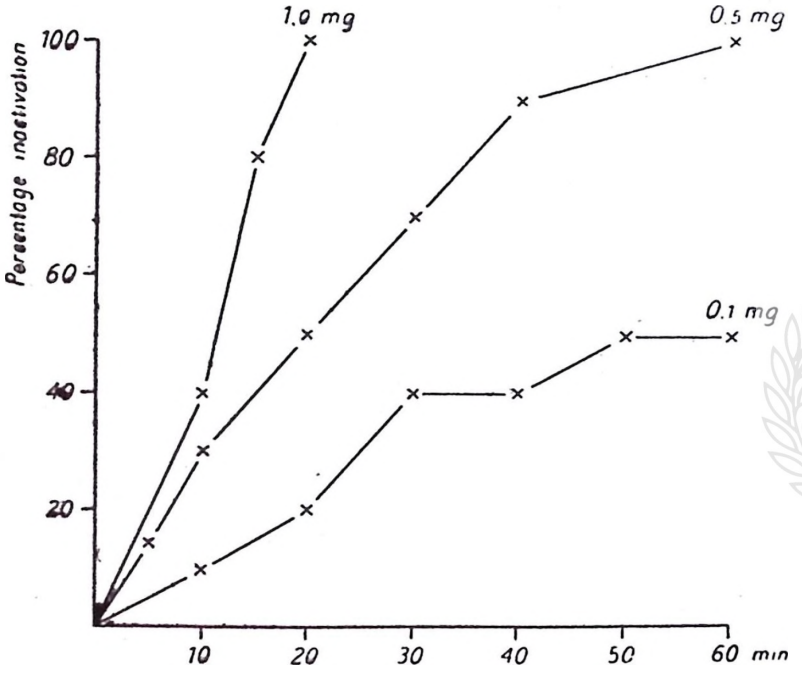


FIG. 1

Inactivation of SP by crystalline chymotrypsin. 0.5 mg of SP (100 U.) in Tyrode's solution was incubated with 0,1—200 µg trypsin in 1—35 min. at 38° C.

STÜRMER: We did not use silicone-treated glassware.

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U. S. v. EULER

DISTRIBUTION AND ACTION OF SUBSTANCE P IN FISH AND INVERTEBRATES

A knowledge of the distribution of a biologically occurring, active substance may often be of aid in understanding its function. This applies to the distribution in the animal series, the macrodistribution in various organs, as well as its microdistribution in the cell. It may be particularly important for substances with largely unknown functions like SP, occurring characteristically in intestinal smooth muscle and in nervous tissue.

The distribution of SP in the animal series is also of interest from the points of view of comparative physiology and may give valuable hints as to evolutionary mechanisms. If its first occurrence in an evolutionary chain can be correlated to a new function or requirement necessitated by altered environmental conditions or prompted by the need for greater achievements, particularly as regards the nervous system, some light may also be shed on its function in the more developed species.

For this reason some studies have been performed on fish and invertebrates (Euler and Östlund 1956, 1957 a, b; Dahlstedt et al. 1959) in order to obtain more information about the occurrence and distribution of SP in cold-blooded animals, since most studies have been made previously on material from warm-blooded animals.

Of previous studies in this field may be mentioned the finding by Correale (1956) that SP occurs in the brain of the frog in high amounts up to 250 U./g.

Material and methods

Preparation of extracts. — As material for the studies to be reviewed here the brain and intestine of teleosts and elasmobranchs have chiefly been used. In some instances extracts have been made of various portions of the brain or the spinal cord. Certain marine invertebrates have been extracted in total or in parts.

Extracts have been made by boiling the organs, after cutting into smaller pieces, in 5 volumes of water to which sulphuric acid was added to pH 4. This reaction was maintained by adding more sul-

phuric acid if necessary. After 10 min. boiling the extract was cooled and filtered on paper. The residue was washed with 1 volume of acidulated water. To the combined extracts ammonium sulphate was added to saturation according to the method of Euler (1936) suggesting its polypeptide nature. The precipitate was allowed to develop at + 5°C overnight, and was filtered off on paper. After repeated washing with 2/3 saturated ammonium sulphate, excess liquid was pressed off and the precipitate dried and powdered.

The further purification was performed according to Pernow (1953) by dissolving the powder in 4 volumes of water under heating to about 50°C and stirring. To the solution was added slowly 2.2 volumes of methanol under continuous stirring. The massive precipitate was allowed to settle in the cold overnight and was washed with 70 per cent methanol. It contained very little SP activity and was removed by centrifugation and discarded. The clear brownish solution, containing practically all of the SP activity, was passed over a column of aluminium oxide (10 g, diameter of column 2 cm, flow 1 ml per minute). The column was eluted with 20 ml portions of methanol in water in falling concentrations from 60 per cent to 20 per cent, followed by repeated elutions with water.

Bioassay. — The methanol present in the eluates was removed by evaporation in vacuo. The aqueous eluates were tested directly. All portions were assayed on the isolated guinea pig ileum according to Pernow (1953) and in most cases also on the isolated rabbit duodenum, the rabbit's blood pressure and the chicken rectal caecum, using purified preparations from cow intestine (100—1,000 U./mg) as standard, prepared according to Pernow (1953).

The specificity of the action was tested

(1) by parallel assays with the standard on different test preparations (Fig. 1) after treatment with atropine and an antihistaminic as outlined above,

(2) by incubating the samples and the SP standard with trypsin (Euler 1936) and comparing the rate of disappearance of action.

Test for biological action in fish. — The biological action of SP preparations from fish intestine and other sources were tested on isolated pieces of small intestine from the teleosts *Pleuronectes platessa* and *Labrus berggylta* and from the elasmobranch *Raja batis*.

The segments of intestine used were suspended in a 75-ml bath with deep sea water diluted 1:2 or 1:2.5 with distilled water for teleosts and 3:5 for elasmobranchs. To the latter solution was added 30 g urea per liter. All experiments were made at room temperature. The drugs were added directly to the bath.

In some instances the intestinal segment was suspended in air and washed with the bath solution at frequent intervals. Drugs were then applied by allowing a small volume of the bath solution, containing the

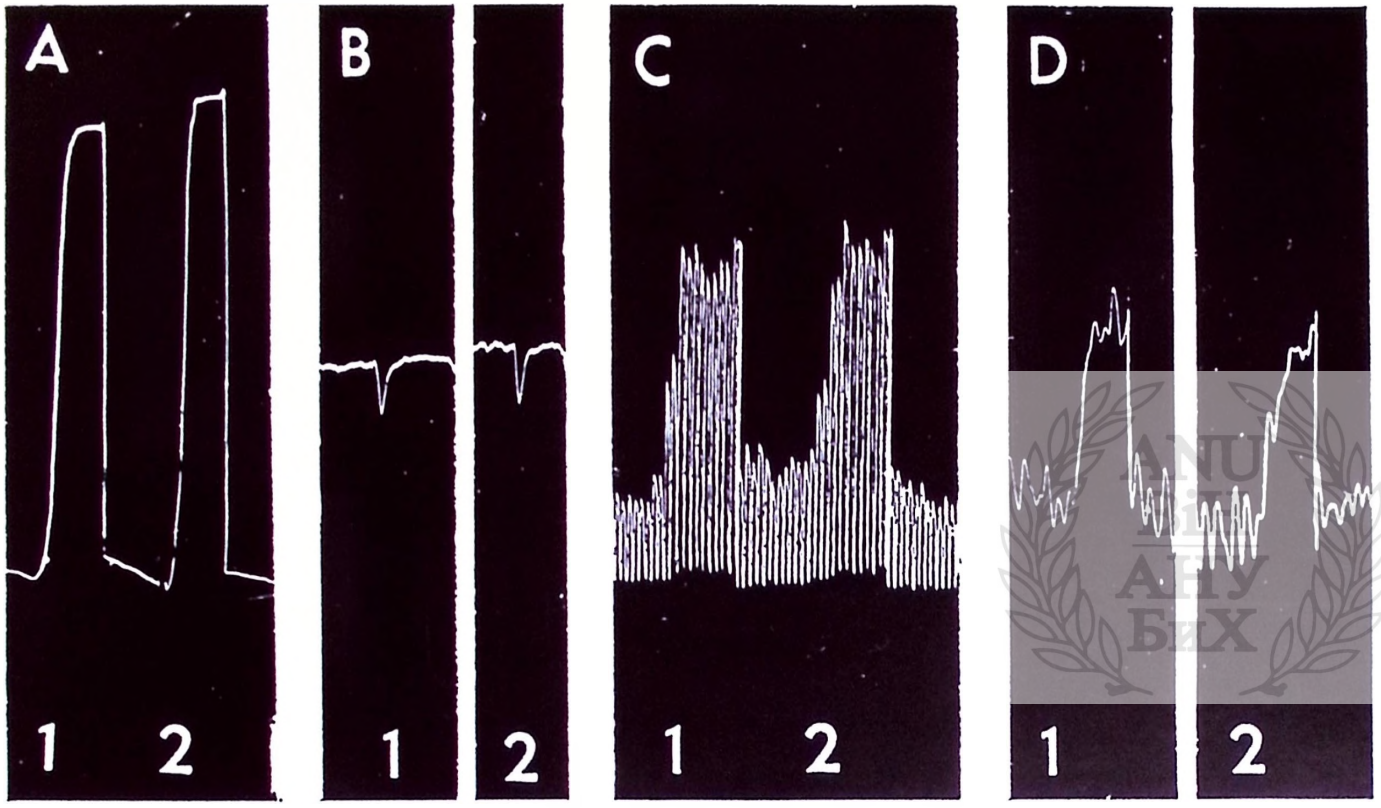


FIG 1

Effects of a purified extract of cod intestine compared with a standard prepared from beef intestine. **A.** Isolated guinea pig ileum, 3-ml bath, 38°C. Atropine and Lergigan (R) 1:2.5 mill. in Tyrode's solution. 1, 0.2 U. SP-standard. 2, 8 ml purified cod intestine extract »C«. **B.** Blood pressure, rabbit, atropine 1, 0.06 ml »C«, 2, 2 U. SP standard. **C.** Isolated rabbit jejunum, 15-ml bath, atropine 1:2.5 mill. 1, 1 U. SP standard. 2, 0.04 »C«. **D.** Isolated chicken rectal caecum, 15-ml bath. 1, 3 U. SP standard. 2, 0.09 ml »C«. [modified after Euler and Östlund (1956) Brit. J. Pharm. 11, 323.]

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drug, to flow directly on the isolated intestine in air, in a way similar to the superfusion technique of Gaddum (1953).

Results

Distribution. — As reported previously SP was found regularly and in relatively high amounts in intestine, brain, and spinal cord of teleosts and elasmobranchs and in smaller amounts in the cyclostome *Myxine glutinosa*. Table I gives some figures obtained.

TABLE I
AMOUNTS OF SP IN FISH ORGANS, UNITS PER GRAM.

	Teleosts		Elasmobranchs		Cyclostome
	Gadus callarias	*Esox lucius*	*Squalus acanthias*	*Raja batis*	*Myxine glutinosa*
Small intestine	5.5	0.7	4.8; 2	3	0.04
Ventricle				1.3	
Whole brain	6			4—9.6	3.3
Telencephalon			50	5.1	
Mid-brain and cerebellum			21	8.5	
Medulla oblongata			45	39	
Spinal cord			18	19—28	
Spinal cord, dorsal part				25	
Spinal cord, ventral part				15	

The following invertebrates were extracted in total and analysed for the presence of SP: *Mytilus edulis*, *Actiniaria* and *Ciona intestinalis*. In the two first-named species no activity which could be ascribed to SP was found in the purified extracts while *Ciona intestinalis* contained a small amount, 0.02 U./g, which corresponded to the effects of SP.

In the intestine of *Nephrops norwegicus* no SP action was found.

Effect of SP on fish intestine. — In view of the presence of SP in extracts of intestine of teleosts and elasmobranchs it appeared

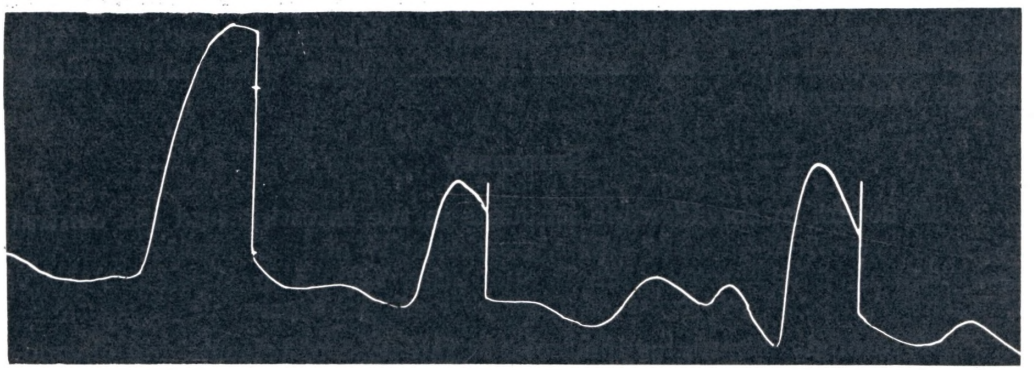


FIG. 2
Isolated small intestine of Pleuronectes platessa, mounted in air, intermittently washed with deep sea water 1:2. Temp. 22°C. Topical application of SP. From left: 0.25 U. SP standard in 0.005 ml, 0.1 U. SP standard in 0.01 ml, 10 µg ACh in 0.1 ml [after Dahlstedt et al. (1959) Acta physiol. scand. 47. 124].

of interest to study whether it exerted actions of similar kind as found in mammals.

It was found that SP, even in small amounts, had a stimulating effect on the isolated intestine. This was particularly obvious when the superfusion technique was applied. Thus 0.1 unit SP in 0.01 ml caused a marked contraction of the isolated small intestine of *Pleuronectes platessa* (Fig. 2). Considering the activity of pure SP (Franz, Boissonnas and Stürmer, 1961), which was found to be 30,000—35,000 U./mg, the total amount given in the case mentioned would correspond to 3.3 ng.

Discussion

The studies reported so far on the presence of SP in fish indicate that it is present in considerable amounts in the intestine and in the central nervous system in teleosts and elasmobranchs. The high amounts in the brain and spinal cord of elasmobranchs are particularly noteworthy.

In the cyclostome investigated, *Myxine glutinosa*, the amounts were smaller, especially in the intestine. It is tempting to relate the low content of SP in the hagfish intestine with the low degree of motility of this organ in comparison with the small intestine of teleosts in particular. If SP functions as a motility hormone of mammalian intestine — for which some support can be advanced — it seems possible that it exerts a similar function in fish in view of its strongly stimulating effect on this organ in teleosts and elasmobranchs.

Before the functional significance of SP in the brain and spinal cord of fish can be fruitfully discussed more knowledge is required as to the normal physiology of the CNS of different species and the action of SP on specified mechanisms.

Of certain interest appears the presence of SP in measurable quantities in *Ciona intestinalis*, since this invertebrate has a dorsal chord during a certain stage of its development which may be associated with some arrangement of the nervous system placing this animal closer to the vertebrates.

Summary

SP occurs in considerable amounts in the intestine and the central nervous system of teleosts and elasmobranchs and in smaller amounts in the cyclostome *Myxine*. Small quantities were also found in *Ciona intestinalis*, but so far none in *Mytilus* and *Actiniaria*.

SP strongly stimulates the isolated intestine of teleosts and elasmobranchs.

It is concluded that SP has a relatively wide distribution in the animal kingdom and exerts similar actions on intestinal smooth muscle as in mammals.

RAZDIOBA I DJELOVANJE SP KOD RIBA I BESKIČMENJAKA

SP se u znatnim količinama nalazi u crijevu i centralnom nervnom sistemu teleosta i elazmobranha, a u manjim količinama kod ciklostome Myxine. Male količine SP nadene su i kod Cionae intestinalis, a potpuni nedostatak SP kod Mytilusa i Actinariae.

SP snažno stimulira izolovano crijevo teleosta i elazmobranha. Zaključuje se da je SP široko rasprostranjen u životinjskom carstvu i da djeluje na glatku muskulaturu crijeva nižih životinja, slično kao kod sisavaca.

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DISCUSSION

KRIVOY: We have been able to cause electric catfish to discharge by bathing them in 0.05 U./ml of SP.

LEMBECK: We also did not find SP in avertebrates (molluscs, holoturia). In the different species of vertebrates SP occurs mainly in the central gray matter, the amount in the telencephalon depends on the same extent of the amount of the central grey matter which reaches the fore brain.

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U. S. v. EULER AND F. LISHAJKO

PRESENCE OF SUBSTANCE P IN SUBCELLULAR PARTICLES OF PERIPHERAL NERVES

SP has been shown to be present in peripheral nerves in amounts which are characteristic for different kinds of nerves. Thus Pernow (1953) found amounts varying from 5 units SP/g in the phrenic nerve of dogs to 45 U./g in the dorsal roots. The amounts found in the sciatic nerve of the dog were 10—15 U./g. His results were essentially confirmed by Amin, Crawford and Gaddum (1954). Using butanol-extraction of acetone-dried powder Leach (1959) found lower figures (8.7 U./g) in dorsal roots of cattle.

Little is known so far about the location of SP in the axon. In view of the findings of Euler and Hillarp (1956) that noradrenaline occurs in subcellular particles in adrenergic nerves it appeared of interest to study whether SP might also be present in special structures which may be possible to separate from the rest of the nervous axonal tissue.

Methods

Sciatic and brachial nerves were removed from large dogs immediately after death. The animals had been maintained in chloralose anaesthesia for 1—6 hours. The nerves were freed from the sheath and squeezed between nylon cylinders in the cold according to a technique previously described (Euler 1958). The press juice and washing fluid (0.13 M K-phosphate, pH 7.0) were centrifuged for 5 min. at $600 \times g$ in order to remove larger particles, if occurring. The supernatant was centrifuged for 30 min. at $50{,}000 \times g$ in the cold and the sediment extracted with 1 ml 0.1 N H_2SO_4 and diluted with water to 6 ml. The clear extract was neutralized with sodium hydrogen carbonate to pH 6.2.

Assay. — The extracts were tested on the rabbit duodenum, the guinea pig ileum and the rabbit blood pressure. The majority of assays were made on the guinea pig ileum.

The general course of assay was the following. On the untreated preparation the extract was assayed against ACh, H and, in some cases against 5-HT and SP. After atropinization and treatment with an antihistaminic (Lergigan (R)) the assay was repeated against 5-HT and SP.

Atropine sulphate and Lergigan were added to a concentration of 10^{-5} g/ml in the bath. Higher concentrations often depressed the response to drugs in an unspecific way. The effect of 5-HT was annulled with LSD in a concentration of 10^{-7} g/ml. The biological activity remaining after LSD was tested against SP. The extract was then subjected to incubation with trypsin 0.2 mg per ml for periods up to 45 min. at 37° and pH 7.

During this treatment the activity gradually disappeared like that of SP and along a similar time course. The result strongly supports the assumption that the active substance found in the sediment is in fact SP. The contraction on the guinea pig ileum was often somewhat slower than that of SP, but on several occasions no difference could be noted.

Fig. 1 shows the effect of an extract of the nerve sediment obtained by high *g* centrifugation in comparison with ACh, H, 5-HT and SP standard before and after specific inhibitors and treatment with trypsin. An increase in the response to SP as well as to the extracts was observed after LSD (Fig. 2). An effect of this kind was first described by Krivoy (1957).

When assayed against a standard of SP (75 U./mg, made by Hoffmann la Roche and kindly placed at our disposal by Prof. J. H. Gaddum) the sediment was found to contain approximately 4—8 U./g of nerve. A certain loss in biological activity of the extract was noted after treatment of the test organ with atropine and with Lergigan (R), but not after LSD. This presumably indicates that some of the biological activity on the untreated or partially treated test organ was due to ACh and H. When the effect of the extract was increased after treatment with LSD it was not possible to state whether or not a loss of action due to the presence of 5-HT had occurred.

The loss in activity of the extract of the sediment after Lergigan (R) (4 U./g) corresponds to 1 µg histamine and the loss after atropine (3 U./g) to 0.06 µg ACh. These figures are, however, nominal and are based on the unproven assumption that the relative activities are unaltered and that no other compounds are present which may influence the response. (Table I).

TABLE I

BIOLOGICAL ACTION EQUIVALENTS OF SEDIMENT H IV FROM THE SCIATIC AND BRACHIAL NERVES OF THE DOG, PER G OF NERVE

	Tested directly	After Lergigan (R)	After Atropine	After LSD
H equ. µg/g	3	—	—	—
ACh equ. µg/g	0.24	0.25	—	—
5-HT equ. µg/g	—	—	6	—
SP equ. U./g	12	8	5	6

Equivalents: 1 U. SP = 0.25 µg H = 0.02 µg ACh.

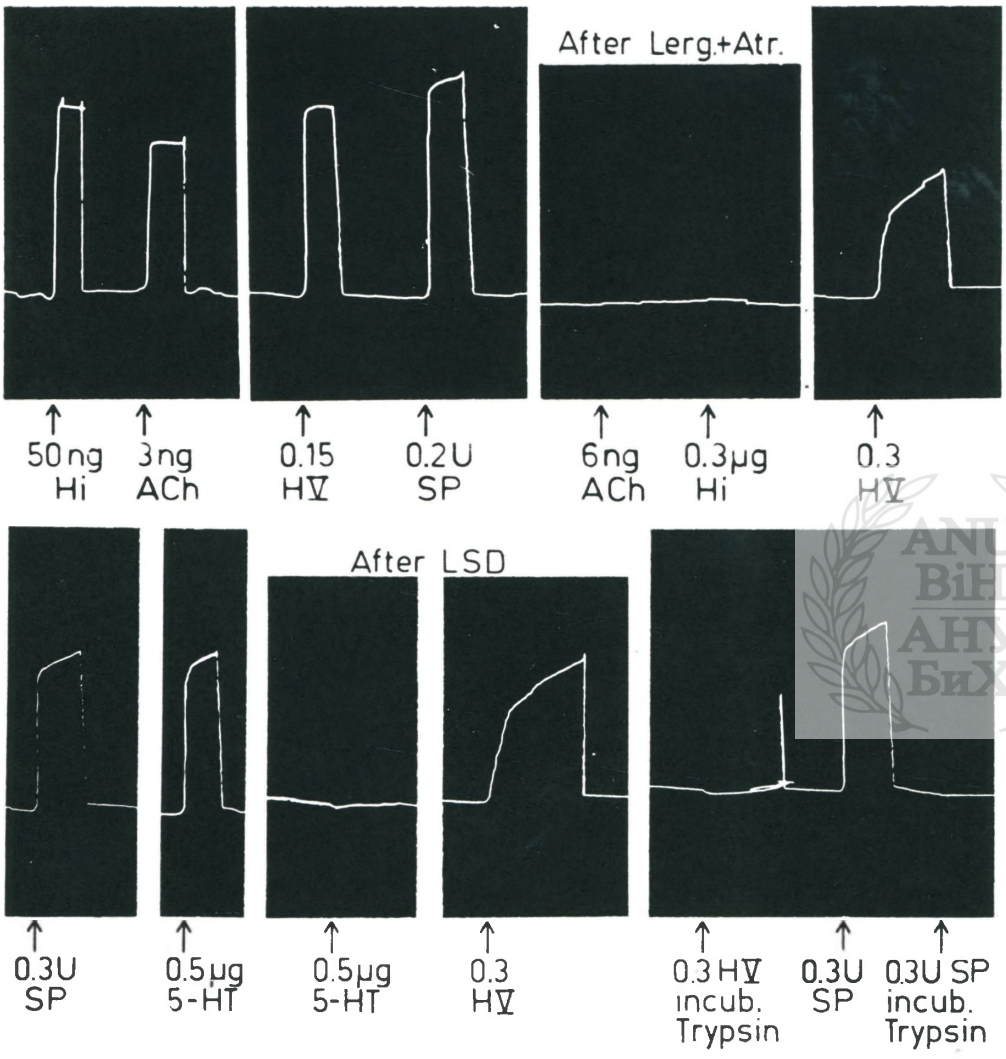


FIG. 1

Isolated guinea pig ileum. 3 ml bath, 38°C, Tyrode's solution. H V, extract of sediment from press juice of sciatic and brachial nerves of the dog, figures representing volumes in ml (1 g nerve = 3 ml).

Drugs administered as indicated (H = histamine, ACh = acetylcholine,, 5-HT = 5-hydroxytryptamine).

Lergigan (R), atropine, and LSD added to the bath fluid reservoir in a concentration of 10^{-7} g/ml.

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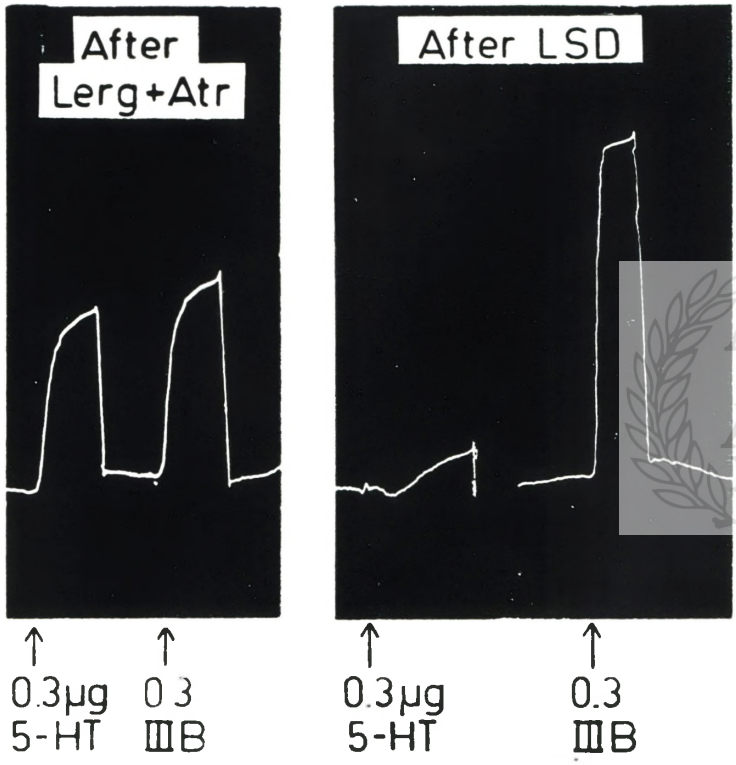


FIG. 2

Isolated guinea pig ileum. Atropine, Lergigan (R) as in Fig. 1. Effect of extract III B, prepared as extract H V, enhanced after LSD.

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Tests on the rabbit intestine and the rabbit's blood pressure were in accord with the assumption that the active substance is SP.

Discussion

The functional role of SP in peripheral nerves is still not understood. Its presence in intraaxonal granules is, however, of interest since it suggests that the polypeptide is stored in a specific way and may be functionally related to the mechanisms of nerve action. The SP in the sedimented granules occurs in a bound or protected form since addition of the untreated sediment to the organ bath has a much weaker action than after extraction with acid. This is in agreement with the catecholamine stored in granules of adrenergic nerves (Euler 1958). The proportion of SP found in the granules is fairly high, and may be estimated at about 50 per cent of the total amount. The general occurrence of SP in nervous tissue suggests an action which may be related to some function of a more general nature or to the metabolism of the nerve. Its presence in the nervous system of fish seems to support this concept.

Summary

The subcellular sediment obtained on high *g* centrifugation of press juice from the sciatic and brachial nerves of the dog contains an active factor having biological actions and chemical properties suggesting its identity with SP, as shown by the action of specific inhibitors and treatment with trypsin.

The active substance is present in the sediment in a bound or protected form or can be released by extraction with acids.

The amounts of the SP-like substance found in the sediment is 4—8 U./g of nerve, indicating that up to 50 per cent, and perhaps more, is present in a granular fraction.

The loss of biological activity of the extract of the sediment after treatment with specific inhibitors suggests the presence of H and ACh in small amounts.

The presence of SP in a bound form in the neurons points to some action of SP in the general function of the nerve.

PRISUTNOST SP U SUPCELULARNIM ČESTICAMA PERIFERNIH ŽIVACA

Supcelularni sediment dobiven prilikom ultracentrifugiranja istiještenog soka n. ischiadicus i n. brachialis psa sadrži faktor čija biološka i kemijska svojstva sugeriraju identitet sa SP, kako pokazuju djelovanja specifičnih inhibitora i obrada tripsinom. Aktivna supstancija u sedimentu nalazi se u vezanoj ili zaštićenoj formi, iz koje se može osloboditi ekstrakcijom pomoću kiselina. Količine supstancije slične SP nađene u sedimentima iznose 4—8 jed./g tkiva, što pokazuje da je do 50% te količine, a, možda, i više, u granularnoj frakciji. Gubitak biološke aktivnosti ekstrakata poslije obrade specifičnim inhibitorima sugeriše prisutnost malih količina H i ACh. Postojanje SP u neuronima u vezanoj formi ukazuje na izvjesnu ulogu ove supstancije u općoj nervnoj funkciji.

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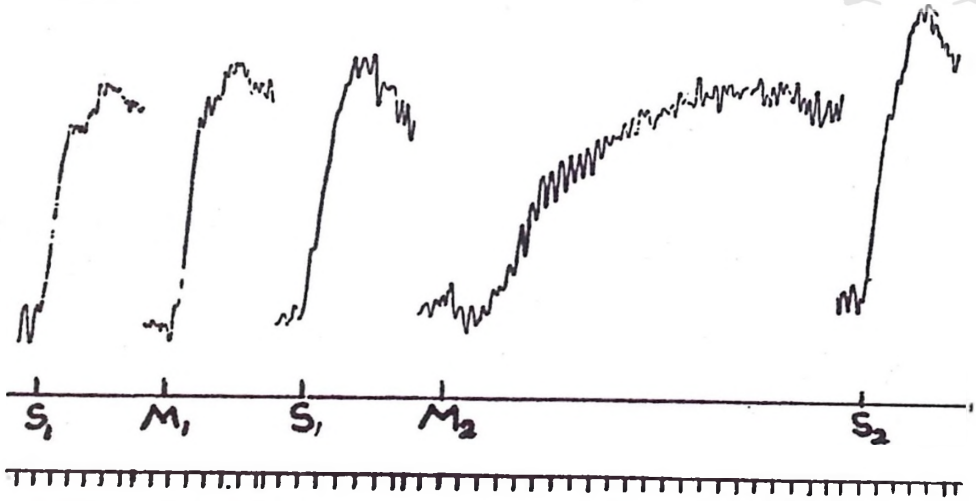
DISCUSSION

GADDUM: Am I right in supposing that these residues contained both microsomes and mitochondria? C. O. Hebb found that cholinacetylase in the CNS was in the mitochondrial fraction, whereas cholinacetylase in the sciatic nerve was in the microsome fraction. It would be interesting to know whether there is a similar difference for SP.

v. EULER: We have not studied in particular the presence of mitochondria and microsomes in the sediment of nerves containing SP but there is little doubt from experiments with splenic nerves that they are present.

ZETLER: The finding that the microsomal fraction of peripheral nerves is as such without biological activity unless it is treated with sulphuric acid contrasts to what I have seen with mitochondria prepared from mouse brain. The experiment shown in the following figure demonstrates that substance P can easily be extracted from mitochondria by boiling at pH 3 (HCl), furthermore, that the same amount of biological activity is very slowly released if the corpuscles are, without pretreatment, suspended in the bath fluid.

It can be assumed that substance P is present in brain mitochondria in an active form and is released during the breakdown of the mitochondrial membrane in Tyrode's solution at 32°C. Thus, substance P seems to be stored in the central nervous system and peripheral nerves in different ways. The small fraction of substance P present in mouse brain which cannot be extracted by mild methods [Zetler G. and G. Ohnesorge (1957) — Naunyn-Schmiedebergs Arch. exp. Path. Pharmak. 231, 199] may also be stored in a way as suggested by Euler and Lishajko.



Isolated guinea pig ileum, bath volume 3 cm³, temperature 32°C (atropine 10^{-7}, mepyramine 10^{-9}, tryptamine $2/10^{-5}$, S_1 : 0.2 U. substance P, S_2 : 0.24 U. substance P. M_1 : 0.1 cm³ mitochondrial fraction of mouse brain, 3 minutes boiled at pH 3 (HCl) and neutralized with NaOH; M_2: the same dose of mitochondrial fraction, but neither acidified nor boiled. The doses of M_1 and M_2 correspond to about 3.5 mg mouse brain each (the cerebellum was removed before mincing). The time marker indicates 10 seconds.

DANICA KOCIĆ-MITROVIĆ

SUBSTANCE P IN PREGNANCY

The localisation of SP in the organism is well known. It is found in extracts of many organs from different animal species, mostly in brain, intestine and retina, though in other organs too, in smaller amounts [Euler and Gaddum (1931), Douglas, Feldberg, Paton and Schachter (1951), Pernow (1953), Duner, Euler and Pernow (1954)]. During the thirty years following its discovery the main outlines of chemical properties and physiological action of SP have been covered in a series of papers. We know that SP exerts, besides from other action, some central effects (Euler and Pernow, 1956); probably it also acts as a transmitter substance between the first and second sensory neurons (Lembeck, 1957), as a motility hormone in the digestive tract (Ohnesorge, 1957), and, finally, it is assumed that SP is a physiological tranquillizer (Stern, 1958). Because of the multiple and important functions attributed to SP it was interesting to investigate its effects in pregnancy, the more so, since analogous investigations have been undertaken earlier with a related polypeptide, synthesized in almost the same regions of the central nervous system as SP, namely oxytocin.

We proceeded first to estimate the concentration of SP in the brain of the pregnant rat. SP was extracted following the method by Zetler and Schlosser (1955). A non-pregnant female from the same litter was used as a control. SP in the extracts was estimated on isolated guinea pig ileum after blocking the ACh, H, and 5-HT receptors by atropine, phenergan and LSD, respectively. The contractions were the characteristic SP-type.

In these experiments we observed a marked decrease of SP concentration in pregnancy with particularly low values in its second half (Fig. 1). This finding gave rise to a new problem: why is SP lost during pregnancy and where does it go? This problem is not only interesting per se, but also in connection with such facts as the increase of histaminase (Marcou et al., 1938) and oxytocinase (Tuppy H., 1960) activity in pregnancy, and in view of the incapability of either cerebrospinal fluid or blood to destroy SP (Gullbring, 1943). The answer to these questions was sought in another series of experiments. A standard SP solution (5 ml; 10^{-3} U./ml) was incubated for 1 hour in

a water bath at 37°C with: (a) two drops of blood serum from a pregnant rat, (b) two drops of non-pregnant rat serum, and (c) without serum (control). After incubation the solutions were tested on isolated guinea pig ileum. These experiments demonstrated a decrease of SP activity after incubation with pregnant serum, but no change after incubation with non-pregnant serum (Fig. 2), and finally that the SP-control during incubation does not change its activity.

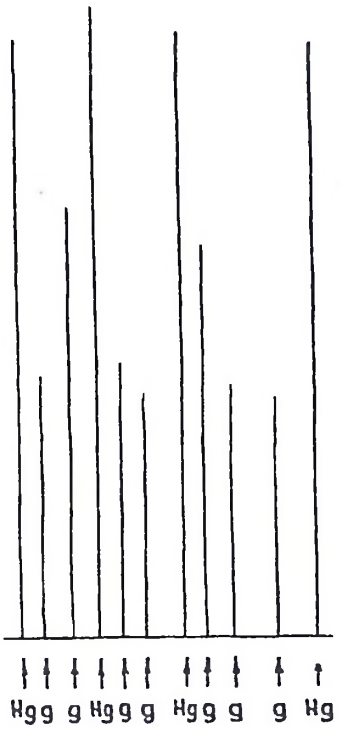


FIG. 1

Concentration of SP in the CNS of pregnant (g) and non-pregnant (Ng) rats.

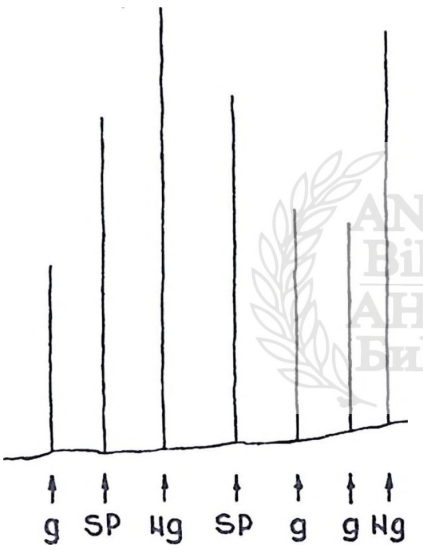


FIG. 2

Concentration of SP incubated with the serum of pregnant (g) and non-pregnant (Ng) rats.

We conclude from these results that, during pregnancy, the SP levels in the CNS of the rat are lowered.

Since SP, according to Lembeck (1956), Holton (1960), and Serafimov (1958), probably takes part in the transmission of sensory impulses we suppose that our results might be interpreted thus, that decreased perception of exogenous stimuli (eventually due to decrease of SP activity) serves to prevent disturbances in the normal course of pregnancy. Our results are in agreement with those of Stern, Gašparević and Kovač (1961) who found that SP is destroyed in human pregnant serum by a factor which is not identical with either diamin-oxidase or vasopressinase or oxytocinase.

Summary

(1) The concentration of SP in the brain of the rat decreases during pregnancy.

(2) The pregnant rat serum contains a factor destroying SP, which is different from diaminoxidase, vasopressinase, and oxytocinase.

(3) We assume that our results could be of some importance in a study of the decreased perception to exogenic stimuly in pregnancy.

SP ZA VRIJEME TRUDNOĆE

Koncentracija SP u štakorovom mozgu i maternici opada u toku trudnoće.

Štakorov serum sadrži za vrijeme trudnoće neki faktor koji uništava SP, a razlikuje se od diaminoksidaze, vazopresinaze i oksitocinaze.

Pretpostavlja se da bi ovi rezultati mogli biti od izvjesne važnosti za proučavanje sniženja percepcije egzogenih podražaja za vrijeme trudnoće.

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DISCUSSION

LEMBECK: How many units per gram of SP have been found in the uterus? Furthermore, it should be interesting to refer the SP content of uterus also to the dry weight.

KOCIĆ-MITROVIĆ: I am afraid I cannot answer this, for I have not worked quantitatively.

LISSÁK: May I ask whether you made a control experiment with progesteron injected to pregnant rats?

KOCIĆ-MITROVIĆ: No, I have not.

VOGT: Is it known whether the total SP-content rises or falls in the pregnant uterus.

KOCIĆ-MITROVIĆ: It rises.

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K. LISSÁK AND E. ENDRŐCZI

THE INHIBITORY SUBSTANCE OF THE BRAIN

In the past years publications in the literature as well as investigations by the present authors have shown that brain tissue contains a substance which may be responsible for nervous inhibitory processes. In their earlier studies the authors have stated that this substance is able to inhibit the ACh sensitivity of peripheral muscle receptors, ganglionic transmission, and, if applied locally, the excitability of the spinal cord and that of the cortex (Lissák and Endrőczi, 1949, 1955, 1956, 1957).

Concerning the vast literature I refer to the subject-matter of two International symposia (Roberts 1960, Florey 1961). In the present lecture I wish to give a picture of our own results, obtained during the last year with an extract from brain tissue, as well as with γ-amino-butyric acid (GABA), and with the biological effects of γ-amino-β-hydroxy-butyric acid (GABOB).

The extract was prepared partly from dog's brain and partly from ox brain following the method used by us in earlier experiments. Upon addition of one volume of 96% ethanol to the freshly removed tissue this mixture was homogenized and then, after addition of one more volume of ethanol and one volume of colloidal aluminium hydroxyde, centrifuged. The transparent supernatant, slightly yellow in colour, was concentrated »in vacuo« to 1/10 of its original volume, clarified with active charcoal, concentrated, then filtered, and the filtrate evaporated »in vacuo«. The residue was redissolved in 10 to 50 ml aqueous ethanol (1:1 v/v) and, after repeated treatment with active charcoal filtered and again evaporated »in vacuo«. Depending on the initial amount of the substance, the residue was dissolved in 1 to 10 ml of 50% ethanol, and then run descending on Whatman No 4 paper with a mixture of phenol-water and/or with butanol : glacial acetic acid : water (4 : 1 : 1 by volume). Different amounts of GABA and GABOB were run as tests and spotted with ninhydrin. After colour development and elution with ethanol : 0,1 N NaOH (4 : 1 v/v), estimation of the GABA content in the brain tissue was carried out photometrically with standard GABA. It should be mentioned here that a series of qualitative chemical reactions were carried out on the paper chromatograms of brain tissue extracts. Among these the for-

mation of picrate in an alkaline medium deserves to be mentioned. As it can be seen in Fig. 1, the picrate-positive area in the system butanol : acetic acid : water shows up at a position corresponding to that of GABA. I wish to mention here that in a different chromatographic system, e. g. in phenol : water, the ninhydrin-positive GABA, too, was situated similarly, relative to the picrate-positive substances in the brain tissue. The importance of this observation cannot be decided yet. However, it was the same area that, by biological testing, proved to possess inhibitory activity.

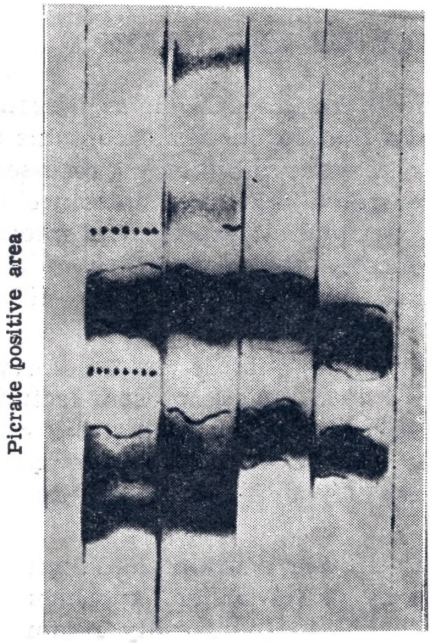


FIG. 1

Paper chromatogram of brain extract: two left stripes indicate the GABA and picrate positive area, two right ones the position of standard GABA and GABOB.

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I shall now pass on to the description of results, suggesting that the brain extract contains an inhibitory factor, but that GABA and/or GABOB can only partly be responsible for the inhibitory effect.

(a) According to observations on isolated cat ileum, the extract eluted from the paper chromatograms is able to inhibit ACh contraction at, or below, pH 7, while, at the same time, even several hundred micrograms of GABA or GABOB are ineffective. The GABA-content in the effective brain extract hardly exceeded 10—15 μg, which shows that GABA or its derivative cannot be responsible for the inhibition.

(b) When examining, on the motor cortex of the cat, the electrical threshold-stimulus for the motor reaction of a foreleg, we found that while local application of an amount of brain extract obtained from 1/3 of a dog's brain resulted in marked decrease of excitability, the same effect could not be obtained with GABA or GABOB.

(c) By means of recording the electrical activity of the cat's cortex, we examined the effects of local and systematic administration of the brain extract, and those of GABA and GABOB on strychnine and

metrazol convulsions. The recordings were made under barbiturate (Evipan-Na) or ether anaesthesia, or in succinyl-choline paralysis with artificial respiration under full consciousness. Our observations may be summarized as follows:

(1) Under barbiturate anaesthesia even high concentrations of the brain extract, GABA or GABOB administered either locally or intravenously did not result in a noteworthy effect on the electrical activity of the somatomotor cortex, recorded with bipolar silver »ball-electrodes«. Under superficial ether anaesthesia, or in succinyl-choline paralysis under full consciousness local, as well as intravenous, administration of the extract from 1/3 to 1/2 of a dog's brain resulted in decreased frequency and increased amplitude, which, however, was of a transient nature. Even large doses of GABA or GABOB failed to produce an effect worth mentioning. During metrazol convulsions produced by the intravenous administration of Tetracor, convulsive activity was considerably decreased, or, with smaller doses of Metrazol, inhibited by the extract from 1/2 to 1 dog's brain given a few seconds before Tetracor.

On the basis of Figs. 2 and 3 it can be seen that during, or immediately before, convulsive activity the intravenous administration of the extract results in a transient inhibitory effect. An attempt to produce an inhibition of a similar nature by the administration of 50—200 mg/kg GABA or GABOB also failed. Local application proved to be less effective, however, when it was used, the activity due to metrazol convulsion was often replaced by a regular synchronized activity of a frequency of 2 to 3/sec., which, without use of the extract, could only be observed very rarely and for a very short time. Even local application of GABA or GABOB failed to influence metrazol activity.

In the following part of our experiment we examined the effects of GABA, GABOB and brain extract by recording the superficial negative convulsive activity produced by strychnine. The monopolar leads were recorded in succinyl-choline paralysis under full consciousness. The amplitude of the negative discharges produced by the local application of a 1,0% strychnine solution was decreased by locally administered GABA or GABOB of 1 mg/ml concentration, which confirmed earlier observations on the subject by Purpura, Girado, Smith and Gomez, 1958. A similar result was obtained when brain extract was applied. Intravenously administered GABA or GABOB did not influence the depolarizing dendritic potentials, while the intravenous administration of brain extract not only prevented the depolarizing activity, but also had a transitory hyperpolarizing effect. Similar phenomena were also reported by Iwama and Jasper, 1957, and by Purpura and coll., 1958, although it was only in the case of a blood-brain barrier destroyed by methanol-chloroform and after the administration of large quantities of GABA that those authors observed a similar effect. From the data in Fig. 4 it may be seen that brain extract can turn the initial

depolarizing activity into hyperpolarization. The development of the mechanism is unknown. However, it is highly probable that in this experiment the abolition of the inhibitory effect of strychnine on axo-dendritic hyperpolarization plays an important rôle.

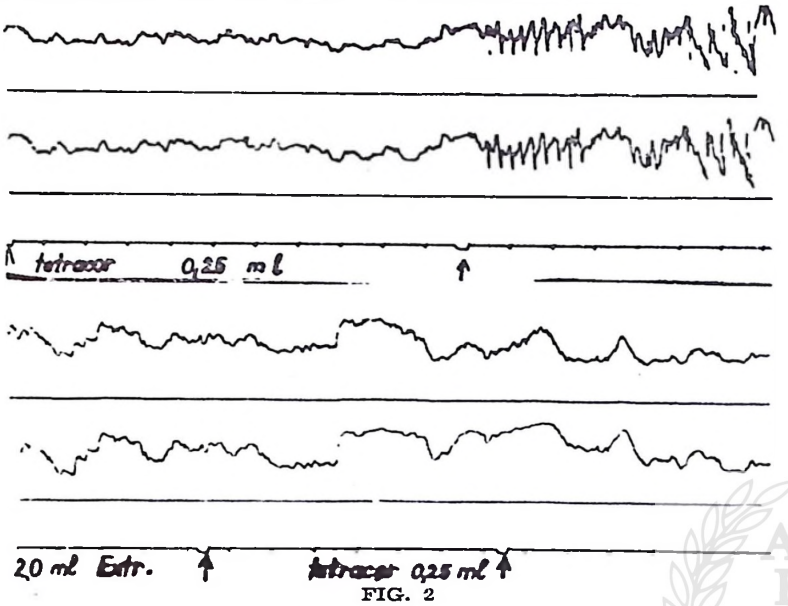


FIG. 2

Metrazol convulsion under the influence of previously administered brain extract. Upper channels : convulsive activity without brain extract. — Lower channels : blocking of the convulsive activity after intravenous administration of brain extract (at arrow).

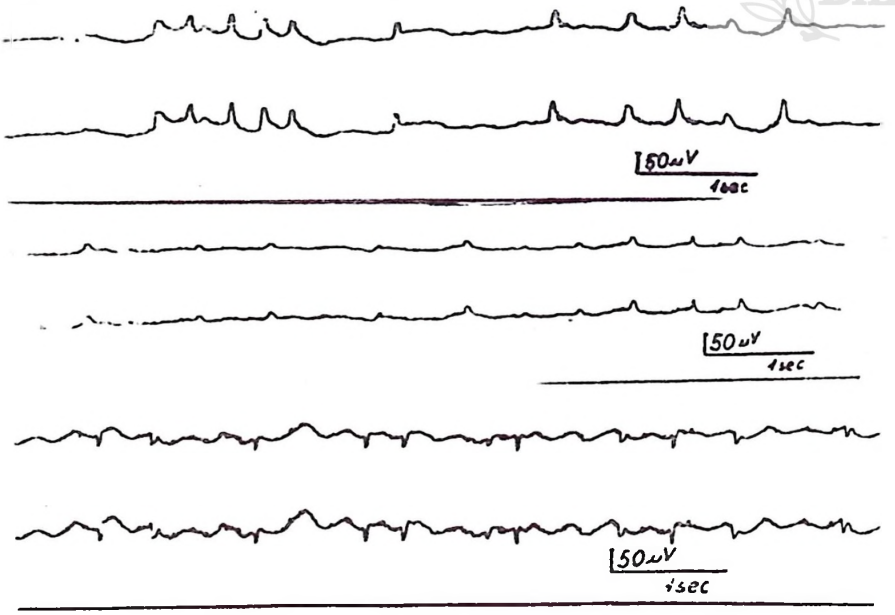


FIG. 3

The blocking effect of brain extract on convulsions induced by metrazol in the cat. Bipolar leads from the somatomotor cortex. The arrows indicate the administration of brain extract (intravenous).

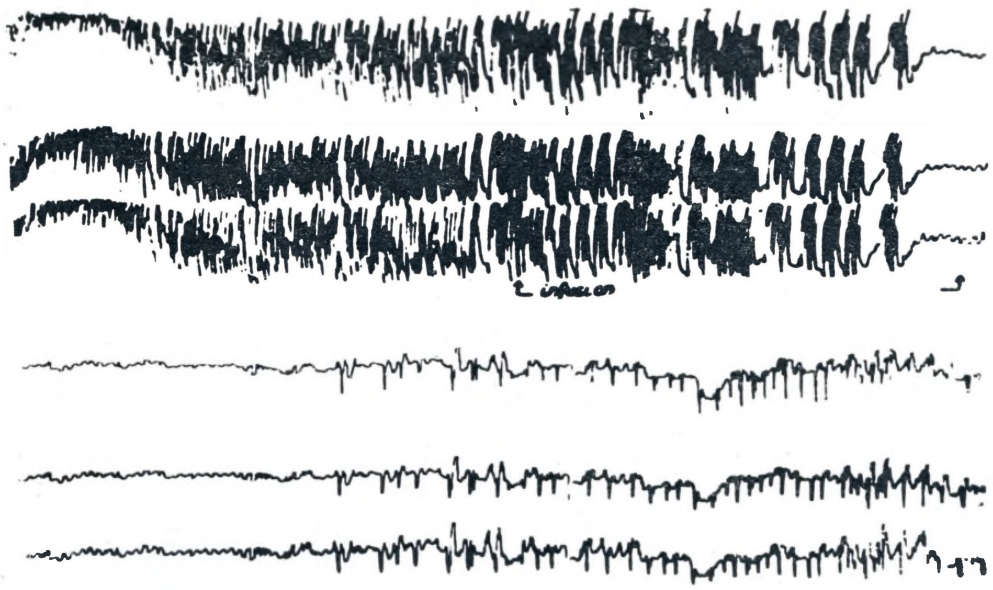


FIG. 4

Electrical activity produced by topical application of strychnine to the animal's brain. Monopolar recording : different silver-ball electrode on the motor cortex, indifferent electrode on the frontal bone. At arrow, surface negative activity was reversed by the intravenous administration of brain extract.

The action of the brain extract and that of GABA on subcortical structures were studied on the conditional reflex activity of dogs with chronically implanted microcannulas. The cannula was implanted stereotaxically, following the methods we usually employ, after the establishment of a conditioned alimentary-motor reflex. In each of the four experimental animals the cannula was localized in the reticular formation and the amount of fluid introduced varied between 0.01 and 0.05 ml. It was observed that the brain-extract strengthened the labile processes of differentiation, and shortened the duration of latency. GABA or GABOB, even in concentrations of 1—10 mg/0.1 ml, failed to produce changes in the conditional reflex processes.

In summary it may be stated that brain tissue contains a substance whose physicochemical properties, as revealed by paper chromatography, resemble those of GABA. However, the two substances differ in their effects. On the one hand, the natural inhibitory substance is more pronounced in its effects, on the other, it will act on receptors on which even high concentrations of GABA are ineffective. The basis of this disparity may be either an essential difference in structure, or the natural inhibitory substance might represent a more complex compound of GABA, which differs in permeability or has a more pronounced biological action.

In order to be able to form a final view of the mediation of inhibition it would be necessary to have a more definite knowledge of the essential properties of the inhibitory factor and the morphologic substrate through which it acts.

Summary

A number of inhibitory phenomena have been observed after administration of extracts prepared from dog's and ox' brain tissue, purified by paper chromatography. It was established that γ-aminobutyric acid (GABA) or its hydroxy-derivative (GABOB) may only partly be responsible for the inhibitory effects. The topical administration of brain extract on the motor cortex resulted in an increase of electric threshold for the motor reaction of a foreleg and a decrease of electric convulsive activity due to intravenously administered Metrazol or to local application of strychnine. The negative discharges elicited by the local strychnine application have been reduced by the brain extract but they were not influenced by GABA or GABOB. The action of brain extract and that of GABA on subcortical structures were studied on the conditioned-reflex behaviour on dogs with chronically implanted canullas. However, the administration of GABA failed to show any effect on the conditioned-reflex behaviour, local application of the brain extract in the mesencephalic reticular formation strengthened the labile processes of differentiation and shortened the duration of latency.

INHIBITIVNA SUPSTANCIJA U EKSTRAKTU MOZGA

Poslije aplikacije ekstrakata dobivenih iz mozga psa i goveda i pročišćenih kromatografijom na papiru, zapažen je niz inhibitivnih pojava. Utvrđeno je da γ-aminomaslačna kiselina (GABA) i njen hidroksilni derivat (GABOB) mogu samo djelomično biti odgovorni za te inhibitivne efekte. Lokalna aplikacija moždanog ekstrakta na motornu koru izazivala je povišavanje električkog praga za motornu reakciju prednjeg ekstremiteta, a snižavanje električke konvulzivne aktivnosti poslije davanja metrazola i. v. ili strihnina lokalno. Negativna izbijanja što ih izaziva lokalna aplikacija strihnina bila su snižena djelovanjem ekstrakta, ali ne i djelovanjem GABA ili GABOB. Djelovanje moždanog ekstrakta i GABA na supkortikalne strukture studirano je na uslovnorefleksnom ponašanju pasa s kronično implantiranim kanilama. GABA je potpuno bez efekta na uslovnorefleksno ponašanje, a lokalna aplikacija moždanog ekstrakta na retikularnu formaciju moždanog debla pojačavala je labilne procese diferencijacije i skraćivala period latencije.

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A. CARRARO, F. CLEMENTI, F. FRASCHINI, L. MARTINI
AND E. MÜLLER

NEUROHUMORAL CONTROL OF THE ANTERIOR PITUITARY GLAND

Developments of the last few years have made it increasingly clear that the nervous system may be considered a complex endocrine system. The view is now developing rapidly that neurons may be producers and releasers of a variety of active physiological agents known as neurohumors. Some nervous cells, termed »neurosecretory cells« (Scharrer, 1959) have carried the secretory activity to the point where they become morphologically distinguishable from other nervous cells.

Substances related to nervous activity which have been chemically identified are ACh, adrenaline, noradrenaline, 5-HT (serotonin) and three posterior pituitary peptides : vasopressin, oxytocin and arginine-vasotocin (Pickering and Heller, 1959; Sawyer, Munsick and Van Dyke, 1959). Neurohumors which are still waiting to be chemically identified are the factors which control aldosterone secretion and those which regulate the activity of the anterior pituitary.

Much evidence has accumulated in recent years to show that the central nervous system influences the secretory activities of the anterior lobe of the pituitary gland (Harris, 1955).

Although the question of innervation of the adenohypophysis is not settled, there is little evidence that important nervous connections exist between it and the brain; it is then likely that nerve fibres which arise in the hypothalamus liberate some humoral substance(s) into the capillaries of the primary plexus of the portal vessels at the level of the median eminence, and that this (these) substance(s) is (are) carried by the portal vessels to the *pars distalis* (Harris, 1955; Assenmacher and Benoit, 1958).

Identification of the ACTH releasing portal chemotransmitter has been claimed by several groups of investigators but the results are up to now conflicting.

A component of SP has been shown to induce ACTH discharge from the pituitary both »in vitro« (Guillemin, Hearn, Cheek and Housholder, 1957) and »in vivo« (Swingle, Parlow, Brannick and Barret, 1956) and then proposed as the possible chemotransmitter involved in the ACTH-releasing mechanism (Pernow, 1953).

Vasopressin (antidiuretic hormone, ADH) has also been proposed as the ultimate mediator of ACTH release; the possible role of this substance is supported by the following evidence: (1) the neurosecretory material stored in the neurohypophysis and containing ADH may be depleted by noxious stimuli which also induce ACTH release (Rothballer, 1953; Scharrer and Frandson, 1954; Kivalo and Rinne, 1960); (2) ADH and ACTH are discharged simultaneously after the exposure to stressful stimuli (Mirsky, Stein and Paulisch, 1954) and after the administration of several drugs (adrenaline, ACh, etc.) (Martini and Rovati, 1956; Casentini, De Poli and Martini, 1957); (3) neurogenic stimuli are much less active as ACTH releasers in neurohypophysectomized than in normal rats, if the operated animals are not given ADH (De Wied, 1960); (4) extracts containing the antidiuretic activity of the posterior lobe and synthetic antidiuretic hormones (lysine- and arginine-vasopressin) are effective in inducing ACTH release in normal animals (Martini and Morpurgo, 1955; Martini, De Poli and Curri, 1956; Casentini, De Poli, Huković and Martini, 1959; Rochefort, Rosenberger and Saffran, 1959), in hypophysectomized animals bearing a functional pituitary graft in the anterior chamber of the eye (Martini and De Poli, 1956; Casentini et al., 1959; Martini, De Poli, Pecile, Saito and Tani, 1959), in animals with hypothalamic lesions (McCann, 1957; Jorgensen and Nielsen, 1958; Jorgensen and Larsen, 1960), in neurohypophysectomized rats (Nowell, 1959) and in pharmacologically blocked animals; in pharmacological experiments hydrocortisone (Porter and Jones, 1956; McCann, 1957; Chauvet and Acher, 1959), 9-alpha-fluorohydrocortisone (Casentini et al., 1959), prednisone (Smelik and De Wied, 1958), prednisolone (De Wied and Mirsky, 1959), morphine (McCann, 1957; Smelik, 1959), nembutal-morphine (Guillemin, Nichols and Lipscomb, 1958; De Wied, Bouman and Smelik, 1958; Munson and Leeman, 1958) or chlorpromazine (Sevy, Ohler and Weiner, 1957) have been used; (5) injections of little amounts of arginine-vasopressin into the third ventricle of dogs produce a significant rise in 17-hydroxy-corticosteroid level in adrenal venous blood (Kwaan and Bartelstone, 1959); (6) natural and synthetic neurohypophysial hormones exhibit ACTH-releasing activity in cultures of hypophysial tissue (Saffran, 1959); (7) the ACTH releasing activity and the pressor activity of lysine-vasopressin are altered to the same extent following mild acid and alkaline hydrolysis, iodination and incubation with placental extracts (Sideman and Sobel, 1960); (8) adrenal corticoids inhibit the release of ACTH as well as the release of ADH (McCann, Fruit and Fulford, 1958; Giuliani, Martini and Pecile, 1960).

The present report will study »in vivo« the ACTH releasing activity of SP and of several natural and synthetic peptides with posthypophysial activities.

Methods

Rats of the Sprague-Dawley strain weighing 150—200 g were used. First of all the ACTH releasing effect of two samples of SP

(obtained through the courtesy of Prof. Gaddum and of Prof. von Euler) and of Pitressin, purified lysine-vasopressin, Pitocin and synthetic oxytocin was studied in normal animals by means of the adrenal ascorbic acid depletion method (Sayers, Sayers and Woodbury, 1948); details on the method employed are given in a previous paper of this laboratory (Casentini et al., 1959). Principles which were found active in this test and a few other synthetic peptides which became available in the last few years were then assayed for ACTH-releasing activity using a new method. In this method the release of ACTH is assessed by variations of concentrations of plasma free corticosterone in rats which had been previously injected intraperitoneally with 25 μg/100 g body weight of dexamethasone in order to block aspecific pituitary stimulation. The structure of the principles employed in these experiments is given in Fig. 1. The time schedule of these experiments was as follows:

hr 0 Dexamethasone 25 μg/100 g, intraperitoneally
hr 3.45 Nembutal 3 mg/100 g, intraperitoneally
hr 4.00 Peptides intravenously
hr 4.30 Decapitation and blood collection

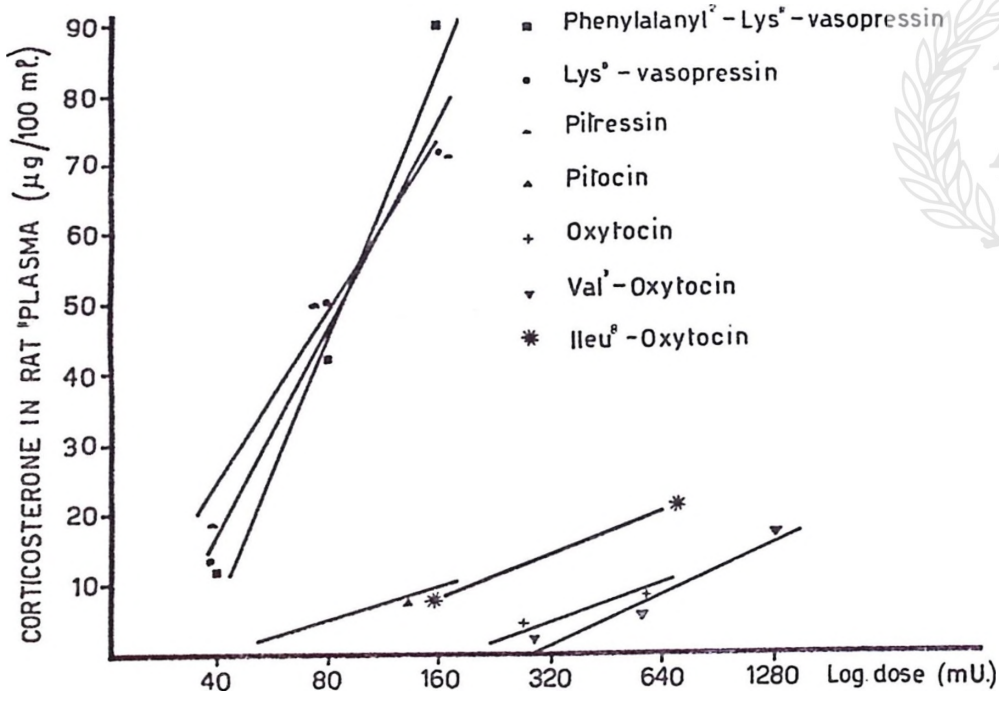


FIG. 1

Administration of dexamethasone has been shown not to interfere with corticosterone blood determinations, to induce a drop of corticosterone blood levels similar to that induced by hypophysectomy and to inhibit the stress induced ACTH-discharge (Giuliani, Martini, Mül-

ler and Pecile, 1961); the sensitivity to ACTH of the dexamethasone-treated animal has also been studied (Giuliani and coll., 1961).

Plasma free corticosterone determinations have been carried out according to the method of Silber, Bush and Oslapas (1958) as modified by Guillemin, Clayton, Lipscomb and Smith (1959).

Results

Table I shows that in normal animals the injection of Pitressin and of Pitocin is followed by a highly significant fall in adrenal ascorbic acid concentration ($P < 0.001$); lysine-vasopressin and synthetic oxytocin also have marked ACTH releasing activity in normal animals; two different samples of SP are completely ineffective in the ascorbic acid depletion test; this last result differs from those obtained by Swingle et al. (1956).

TABLE I

ADRENAL ASCORBIC ACID DEPLETION INDUCED BY VARIOUS PEPTIDES IN NORMAL RATS.

Treatment	No. of rats	Adrenal ascorbic acid concentration (mg/100 g adrenal) Mean ± S. E.	P (Fisher's table)
NaCl 0.9%	26	411 ± 8	
SP (horse) 10 U.	6	427 ± 43	
SP (cow) 10 U.	6	436 ± 28	
Pitressin 0.3 U.	12	302 ± 14	< 0.001
Pitocin 0.3 U.	23	358 ± 12	< 0.001
Lysine-vasopressin 0.3 U.	18	311 ± 8	< 0.001
Synthetic oxytocin 0.3 U.	23	358 ± 16	< 0.005

When the peptides were tested as ACTH releasers in dexamethasone-treated rats the following results were obtained (Fig. 2): Pitressin, synthetic lysine-vasopressin and phenylalanyl-lysine-vasopressin showed a considerable activity; a linear log-dose response relationship could be obtained with doses ranging from 40 to 160 mU.; the greatest activity was shown by the synthetic peptide phenylalanyl-lysine-vasopressin. By contrast Pitocin, synthetic oxytocin, valyl-oxytocin (Berde, Doepfner and Konzett, 1957) and isoleucyl-oxytocin (Berde and Konzett, 1960) were very poor ACTH releasers.

These results seem to indicate that the determination of blood corticosterone levels in the dexamethasone-inhibited rat offers a new approach for the »in vivo« study of ACTH-releasing activity of hypophysiotrophic substances.

Moreover the results obtained with this new test seem to give new support to the hypothesis that posterior pituitary principles may be involved in the physiological regulation of ACTH release.

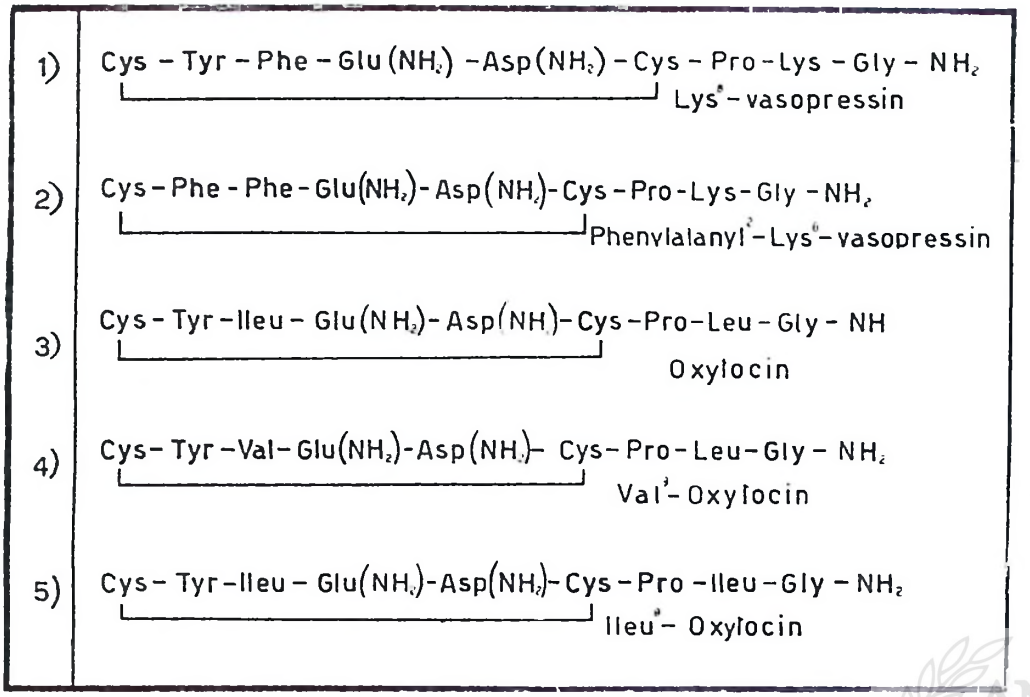


FIG. 2

It seems also worth mentioning that these experiments have clearly shown that also synthetic non-natural peptides may be very active ACTH releasers.

If we consider the acitivity for mg of the peptides used in the present report (which is 280 U./mg for lysine-vasopressin; Du Vigneaud, Bartlett and Jöhl, 1957), it becames evident that these peptides act in very little amounts, ranging from about 0.15 μg to about 0.60 μg. A similar degree of activity was found »in vivo« by Schally and Guillemin (1960) for the preparation they call CRF (Corticotrophin-Releasing Factor).

Similar results and conclusions were also reached by De Wied and Mirsky (personal communication) who have shown that oxypressin, a synthetic peptide which contains the cycle of vasopressin and the side chain of oxytocin, releases ACTH in rats, as measured by the adrenal ascorbic acid method.

A result similar to those reported in the present paper has also been obtained by Miller, Arimura and Dingman (1960) who have shown that lysine- and arginine-vasopressin produce marked increases in plasma corticosterone levels in prednisolone-treated rats and that two unidentified compounds free of vasopressor activity were also devoid of ACTH-releasing activity.

The negative results generally obtained with oxytocin are in agreement with the results of Rinne, Kivalo and Lahtinen (1959) who

have shown that oxytocin is not active in the adrenal ascorbic acid test after prednisolone administration.

Summary

The question whether the hypothalamo-hypophyseal pathway is nervous or humoral in nature has been widely discussed in recent years. The existing evidence points to a humoral rather than a nervous mechanism, and the hypothalamic-hypophyseal portal system has received much attention as the probable pathway through which a chemical transmitter could pass from the median eminence to the adenohypophysis.

Identification of the ACTH-releasing portal chemotransmitter has been claimed by several groups of investigators but the results are up to now conflicting. Two different samples of SP, assayed on the adrenal ascorbic acid depletion test in the rat, have shown to be completely ineffective as ACTH releasers; on the other side Pitressin, Pitocin, lysine-vasopressin and synthetic oxytocin were highly active in this test. Pitressin, synthetic lysine-vasopressin and phenylalanyl-lysine-vasopressin (a synthetic analogue of vasopressin) showed a considerable ACTH-releasing activity when tested in a new method of assay based on corticosterone blood determinations in dexamethasone inhibited rats. By contrast Pitocin, synthetic oxytocin, valyl-oxytocin and isoleucyl-oxytocin (two synthetic analogues of oxytocin) were very poor ACTH releasers in this test.

NEUROHUMORALNA KONTROLA PREDNJEG REŽNJA HIPOFIZE

U toku posljednjih godina mnogo se diskutiralo o pitanju da li je priroda hipotalamo-hipofiznog puta humoralna ili nije. Prikupljeni podaci više govore u prilog jednog humoralnog nego nervnog mehanizma pa je hipotalamo-hipofizni portalni sistem pobudio veliku pažnju kao mogući put za prolaz nekog kemijskog transmitora od središnje izbočine tub. ciner. ka adenohipofizi.

Više grupa istraživača tvrdilo je do sada da su identifikovale portalni kemotrasmitor koji oslobađa ACTH, no dosadašnji su rezultati protuslovni. Pri ispitivanju pražnjenja askorbinske kiseline iz štakorove nadbubrežne žlijezde, pokazalo se da su dva različita primjerka SP potpuno neefikasni za oslobađanje ACTH; pitresin, pitocin, lizin-vazopresin i sintetski oksitocin, naprotiv, bili su vanredno aktivni u istom testu. Pitresin, sintetski lizin-vazopresin i fenilalanil-lizin-vazopresin (jedan od sintetskih analoga vazopresina) pokazali su znatnu aktivnost i pri ispitivanju jednom novom metodom, zasnovanom na određivanju kortikosterona u krvi štakora tretiranih deksametazonom. Za razliku od navedenih spojeva, pitocin, sintetski oksitocin, valil-oksitocin i izoleucil-oksitocin (dva sintetska analoga oksitocina) vrlo su slabo oslobađali ACTH u novom testu.

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DISCUSSION

VOGT: Do you think that the amounts of vasopressin released under physiological circumstances are sufficient to release ACTH, or do you think as do Guillemin and Saffran, that the natural corticotrophin releasing factor is much more potent?

MARTINI: We think that, as it has been pointed out by Mirsky, the amounts of vasopressin released under physiological circumstances are sufficient to release ACTH; our experiments do not exclude, of course, that other molecules related to vasopressin could also be of importance for the nervous regulation of ACTH secretion.

STERN: Milin and I have some evidence that SP could release ACHT. This agrees with the views of some other authors. SP markedly depresses the C-vitamin content of the suprarenal gland.

ANU BiH АНУ БиХ

W. A. KRIVOY

A COMPARISON OF THE ACTIONS OF SUBSTANCE P AND OTHER NATURALLY OCCURING POLYPEPTIDES ON SPINAL CORD

Introduction

Lysergic acid diethylamide (LSD) has been shown to inhibit the enzymatic destruction of SP »in vitro« (Krivoy, 1957; Smith and Walaszek, 1961). This communication represents an attempt to determine if LSD potentiates the actions of SP »in vivo«, thereby providing information on the physiology of SP on the one hand, and the pharmacology of LSD on the other.

Lembeck (1953) suggested that SP is a neurohumor associated with primary afferent transmission in the spinal cord. Although this suggestion was predicated entirely upon the distribution of SP, it seemed most reasonable to initiate this investigation by looking for some interaction between LSD and SP at the level of the spinal cord.

Methods and materials

To evaluate the actions of SP on transmission of nerve impulses along the intramedullary pathway of the primary afferent fiber to secondary and internuncial neurons, use was made of the technique described by Lloyd and McIntyre (1949). These authors described and analysed a series of five potentials which were recorded from a dorsal rootlet adjacent to another rootlet which was stimulated. For the sake of clarity these potentials will be designated according to the nomenclature of these authors (see Fig. 1). The first three dorsal root potentials, DR I, II and III are due to intramedullary conduction of the nerve impulse along the primary afferent nerve. The fourth, DR IV, is in large measure due to a residual negativity associated with supernormality of the nerve endings of the primary afferent fiber, as well as in the secondary neurons. The fifth response, DR V, is associated with supernormality in internuncial neurons (Gasser and Graham, 1933; Rudin and Eisenman, 1953; Eccles and Krnjević, 1959).

Forty-two decerebrate cats were used in this study. All experiments were duplicated on decerebrate cats which had spinal transections at L1. Decerebration and subsequent laminectomy were performed under

ether anesthesia. After exposure of the spinal cord the last lumbar or first sacral spinal roots were dissected and split into two rootlets, each of which was mounted on silver or chlorided silver electrodes. One rootlet was used for stimulating, the other for recording. The entire area of exposed spinal cord and rootlets was covered with mineral oil contained in a trough constructed of the incised skin. The mineral oil was previously equilibrated with CO_2 and maintained at 37°C by radiant heat.

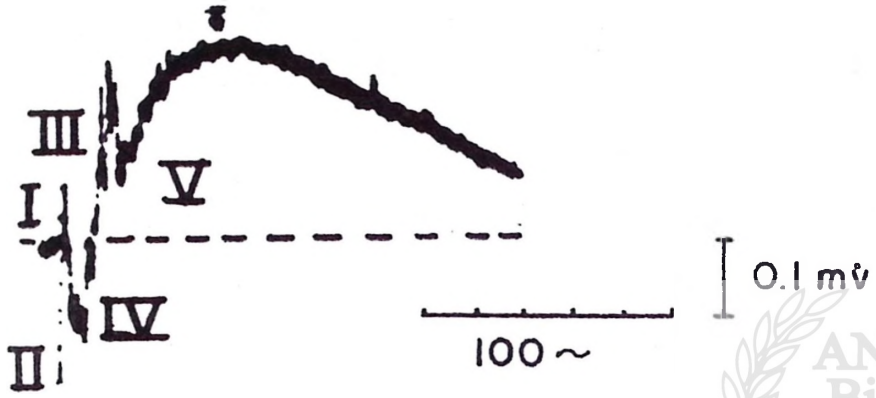


FIG. 1
DR I-V of the dorsal root potential.

Stimuli used were biphasic and either maximal or about 50% of maximal for DR IV. Once stimulation had been started it was maintained at a constant frequency of 0.5 or 2.5 cycles per second (cps) for the duration of the experiment. Stimulation was provided by means of a Grass S4 stimulator isolated from ground by a Schmitt-type stimulus isolation unit (Grass). Preamplification of potentials was accomplished by a Grass P6A DC preamplifier. A Tektronix 502 cathode ray oscilloscope was used for further amplification and display of the evoked potential.

Drugs used in this study were LSD[1], BrLSD[1], SP[2,3], chlorpromazine, synthetic bradykinin[1] and homogenous β-melanocyte stimulating hormone (β-MSH). All drugs were administered intravenously via a polyethylene cannula fixed in the femoral vein. No drug was administered until at least one hour after the termination of ether anesthesia, and then only after the size of the dorsal root potentials had been observed to remain constant for at least thirty minutes.

Blood pressure was monitored in some preparations by means of a mercury manometer connected to the femoral artery. No anticoagulant was used. Respiration was noted by observation.

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[1]) Kindly provided by Dr. R. P. Bircher, Sandoz Pharmaceutical Co., Hanover, New Jersey, U. S. A.
[2]) Kindly provided by Dr. J. H. Gaddum, Institute of Animal Physiology, Babraham, England.
[3]) Kindly provided by D. Graham Chen, Parke Davis and Company, Ann Arbor, Michigan, U. S. A.

Results

A. Substance P. — When submaximal or maximal stimuli of 0.5 cps were used, LSD, SP and combinations of these two drugs were found to have no consistent action on the preparation. When maximal stimuli of 2.5 cps were used, LSD in extremely large doses (70 μg/kg) produced enhancement of DR IV.

When submaximal stimuli were used at a frequency of 2.5 cps, LSD 5 μg/kg was found to enhance DR IV in approximately half of the cats studied, and 10 μg/kg produced enhancement in every cat. When augmentation of DR IV appeared, it was observed within one minute of the time of injection and reached a maximum approximately five minutes thereafter; if at this time the stimulus intensity was reduced such that DR IV was again submaximal, DR IV remained at this new level indicating that the action of LSD had reached a stable level, and that there was no background of continued enhancement.

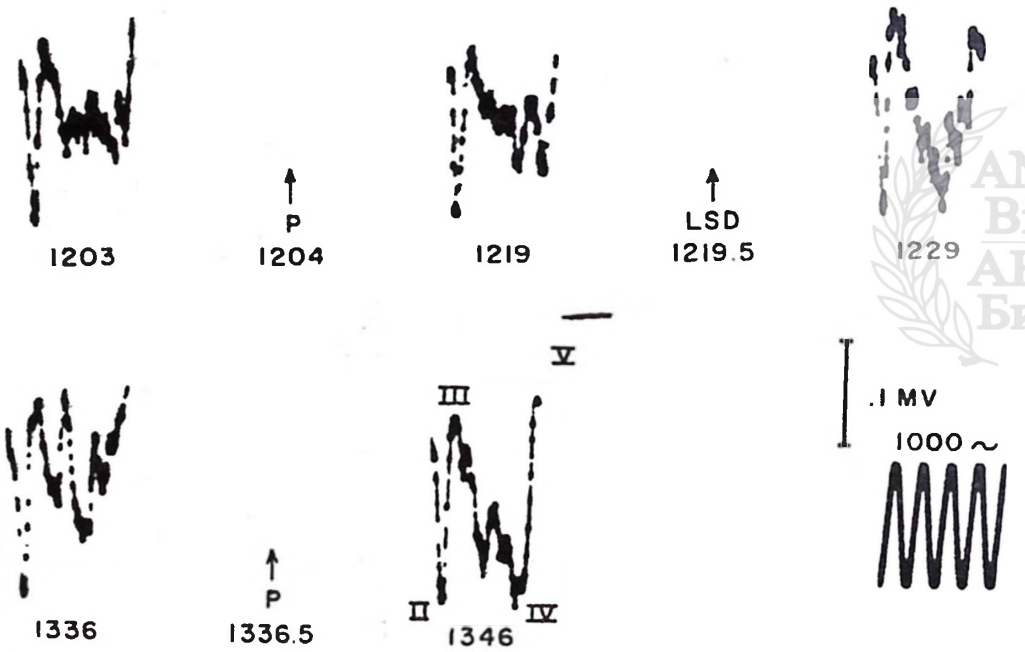


FIG. 2

Dorsal root potentials from a single experiment. The times each record was obtained is indicated below each tracing. Drugs and times of administration are indicated. Injections of substance P (P) (30 U./kg at 1204 and 12 U./kg at 1336.5) and LSD (5 μg/kg) were given via the femoral vein. The Roman Numerals in the final trace indicate the designation of the dorsal root potential sequence. The amplification factor in this experiment was so high that DR V did not appear on the oscilloscope screen. (This figure is reproduced with permission of the Editors of The British Journal of Pharmacology and Chemotherapy).

SP alone was found to have no action on the dorsal root potentials when doses as high as 30 U./kg were injected. On the other hand, under the conditions of submaximal stimulation at a frequency of

2.5 cps, SP was found to have a pronounced action in the presence of LSD, such that when SP, 12 U./kg, was given after 5 μg/kg of LSD, it produced enhancement of DR IV. This response to SP was observed if the previous administration of LSD had not produced its own action, or if, after enhancement was obtained, the stimulus intensity was reduced so as to render DR IV approximately equal to its control value relative to maximal. The enhancement of DR IV under these conditions could not be distinguished from the actions of effective doses of LSD (see Fig. 2). Occasionally DR I, II, III and V were found to increase after DR IV had increased in response to LSD or to LSD followed by SP.

BrLSD in doses up to 100 μg/kg had no action on dorsal root potentials; LSD after BrLSD was found to have no action on dorsal root potentials in doses up to 100 μg/kg.

B. Other Polypeptides. — Because of the observations on interaction between LSD and SP, it became important to know if other polypeptides might modify the dorsal root potentials. Bradykinin was injected and found to have no action. Since Rocha e Silva, Corrado and Ramos (1960) reported that chlorpromazine potentiates the actions of bradykinin and Krivoy and Kroeger (in preparation) reported that chlorpromazine inhibits DR V, it was of interest to determine if bradykinin had any action on the dorsal root potentials in the presence of chlorpromazine. The results of these experiments are indicated in Fig. 3. It was found that bradykinin alone, or in the presence of ineffective amounts of chlorpromazine, had no action on the dorsal root potentials. However, if an effective dose of chlorpromazine was injected, i. e., one which produced depression of DR V, then the subsequent injection of bradykinin was followed by further depression of DR V. In a series of experiments it was found that chlorpromazine induced depression of DR V lasted more than thirty minutes, and the bradykinin-chlorpromazine induced depression lasted more than fifty minutes.

It had been shown previously (Krivoy and Guillemin, 1961) that β-MSH stimulates spinal reflexes. This prompted an attempt to determine if β-MSH had any influence on the depression produced by chlorpromazine or by the combination of chlorpromazine and bradykinin. As can be seen in Fig. 3, the injection of β-MSH was followed by a return of DR V toward the control level. It was particularly interesting that the time course of this recovery followed the time course of spinal stimulation produced by β-MSH (Krivoy and Guillemin, 1961), i. e., the onset of recovery appeared approximately four minutes after injection of the drug, and the maximal action appeared 15—20 minutes thereafter. It was also notable that the dose of β-MSH required to produce this action was approximately 200 μg/kg, whereas in the untreated cat spinal stimulation was seen when doses as low as 2 μg/kg were used.

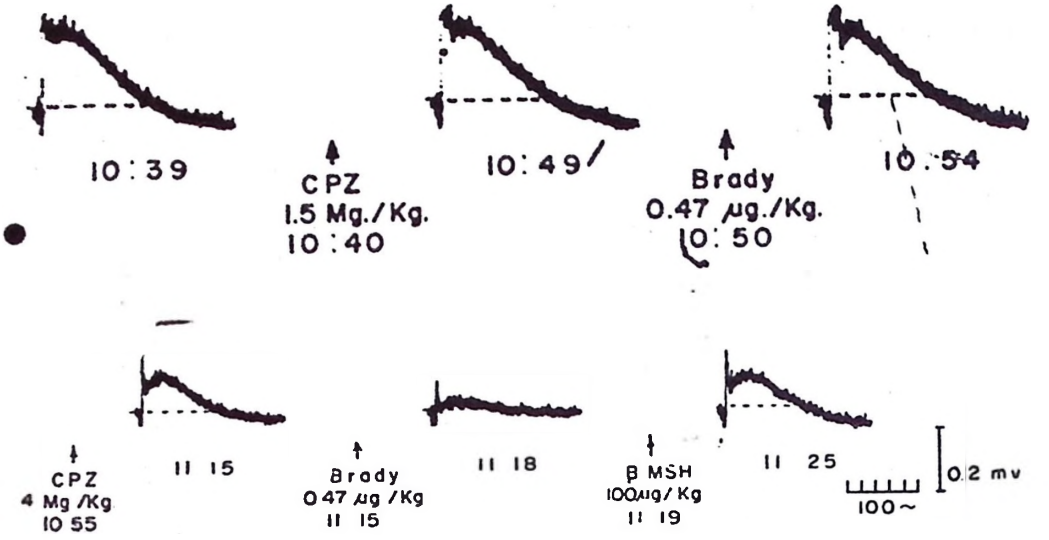


FIG. 3

Dorsal root potentials taken from a single experiment. The time appears below each trace. Injections of Chlorpromazine (CPZ) (1.5 mg/kg at 10:40 and 4 mg/kg at 10:55), bradykinin (Brady) (0,47 µg/kg at 10:50 and again at 11:15), and β-MSH (100 µg/kg at 11:19).

Bradykinin given after LSD had no action on DR IV or DR V. SP, given after chlorpromazine had no action on DR IV or DR V.

There were no observable differences in the responses of decerebrate cats compared with decerebrate-spinal cats. Furthermore, there was no consistant change in blood pressure or respiration which would account for any of the phenomena observed.

Discussion

In view of Gulbring's discovery (1943) of the enzymatic destruction of SP, it is not too surprising that the amounts of SP used here had no action of their own. These data confirm those of Kissel and Domino (1959) that SP has no action on spinal reflexes. Unfortunately, insufficient quantities of SP were available to attempt confirmation of the observation that large amounts of SP inhibit spinal reflexes (Stern and Dobrić, 1957). It is possible that in the spinal cord SP has a diphasic action, stimulating in small concentrations and depressing in larger ones. A diphasic action of SP on ganglia has been observed by Beleslin, Radmanović and Varagić (1960).

The observation that concomitant use of sub-effective concentrations of SP and of LSD produce enhancement of DR IV is predictable, if one accepts Lembeck's conclusion that SP is a neurohumor and at the same time applies »in vivo« the »in vitro« observation that LSD preserves SP from enzymatic destruction (Krivoy, 1957). The fact that large amounts of LSD produce the same phenomena as smaller doses

of LSD plus SP would indicate a system which is analogous to the phenomena observed with the anticholinesterases and ACh.

The finding that LSD was not observed to have an action at low frequencies is in keeping with the concept that it is an enzyme inhibitor. It is known that SP is rapidly destroyed. If LSD acts by preventing the destruction of a neurohumor, then its action would appear most dramatically at a time when the rapid destruction of the neurohumor is most critical, i. e., during a period of rapid stimulation.

Morphine antagonism by large doses of SP (Zetler, 1956) can be explained by physiological antagonism of morphine and SP. We have seen here that SP enhances the flow of sensory impulses (augmentation of DR IV). Krivoy and Huggins (in preparation), have shown that morphine inhibits sensory impulses at a later adjacent site (inhibition of DR V).

BrLSD had no action on the dorsal root potentials. This is in keeping with the finding that BrLSD does not modify the rate of destruction of SP (Krivoy, 1957). On the other hand, the observation that BrLSD inhibits the actions of LSD and of combinations of LSD and SP is of interest because of the report that BrLSD antagonizes the hallucinogenic properties of LSD (Ginzel and Mayer—Gross, 1956).

Neurogenic specificity among the polypeptides as a specific property is borne out by the differential actions of these substances. Under appropriate conditions SP enhances DR IV, bradykinin depresses DR V and β-MSH enhances DR V whereas no neurogenic activity could be demonstrated for α-MSH, vasopressin, oxytocin or ACTH (Krivoy and Guillemin, 1961). Furthermore, since the phenomena observed could be obtained in both the decerebrate cat and in the decerebrate-spinal cat, it would appear that SP, bradykinin and β-MSH act directly on the spinal cord, rather than on some more centrally located nervous tissue. The concept that these drugs act directly on the spinal cord is further substantiated by the fact that there was no correlation between the changes in dorsal root potentials and alterations in respiration or in blood pressure.

The data presented in this paper would tend to support Lembeck's concept that SP is a neurohumor in the primary afferent pathway of the spinal cord. They would also tend to support the concept that if the neurogenically active polypeptides are neurohumors, they are most likely modulators rather than detonators.

Summary

LSD, 10 μg/kg, was found to enhance DR IV of the dorsal root potential sequence of the cat spinal cord. SP alone had no action. Sub-effective amounts of LSD followed by sub-effective amounts of SP resulted in enhancement of DR IV. BrLSD had no observable action on the dorsal root potentials, but antagonized the actions of subsequently administered LSD and combinations of LSD and SP.

Bradykinin was found to have no action on the dorsal root potentials. Chlorpromazine, in adequate concentrations, depresses the dorsal root potentials. Combinations of chlorpromazine and bradykinin produce a depression of the dorsal root potentials which is greater than the action of chlorpromazine alone.

β-MSH is capable of antagonizing the actions of chlorpromazine and of combinations of chlorpromazine and bradykinin.

USPOREDBA DJELOVANJA SP I DRUGIH PRIRODNIH POLIPEPTIDA NA KIČMENU MOŽDINU

LSD u dozi od 10 μg/kg pojačava DR IV-sekvencije potencijala dorzalnog korijena kičmene moždine mačke. SP, sama, bila je nedjelotvorna. Subefektivne doze LSD i, poslije njih, subefektivne doze SP pojačavaju DR IV. Brom-LSD nije imala vidljivog efekta na potencijale dorzalnih korjenova, ali je djelovala antagonistički u odnosu na LSD i kombinaciju LSD—SP, ako su ove aplikovane poslije brom-LSD. Bradikinin ne djeluje na potencijale dorzalnih korjenova. Klorpromazin, u pogodnim dozama, djeluje depresivno na potencijale dorzalnih korjenova. Kombinacija klorpromazina i bradikinina također djeluje depresivno na potencijale dorzalnih korjenova, ali u jačoj mjeri nego sam klorpromazin. Hormon koji stimuliše β-melanocite djeluje antagonistički u odnosu na klorpromazin i bradikinin.

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DISCUSSION

GADDUM: Have you tried giving SP by close arterial injection?

KRIVOY: No.

HUKOVIC: We have seen tachyphylaxis to SP after LSD. What explanation do you think could there be for this?

KRIVOY: The tachyphylaxis to SP can be explained on the basis of professor Gaddum's findings.

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P. STERN, IVANKA GAŠPAROVIĆ AND JULIJANA KOVAČ

A FACTOR INACTIVATING SUBSTANCE P PRESENT IN THE SERUM OF PREGNANT WOMEN

Ever since the discovery of SP, its destruction by specific or unspecific enzymes has been a question of major interest. Such an interest is fully justified in view of the fact that SP is a physiological product, belonging probably to the group of biologically active polypeptides which undergo destruction by specific enzymes. We have reason enough to believe that SP is chemically related to oxytocin and vasopressin, though recent results by Franz, Boissonnas and Stürmer (1961) do not seem to support our opinion. Since SP is produced in tissues rich in oxytocin and vasopressin which in turn, interact with specific enzymes, it seems reasonable also to assume the existence of an enzyme specifically inactivating SP.

The inactivation of SP by various tissues has been well known for a long time. Gaddum and Schild (1934), as well as von Euler (1934) have reported it many years ago. Subsequently Pernow (1955), Arvidsson et al. (1956), Lembeck and Eber (1956) and Matussek (1959) dealt with inactivation of SP in detail. Arvidsson, Pernow and Swedin (1956) have expressed the opinion that there must be a specific SP-ase. These authors succeeded in separating an SP inactivating component from diaminooxydase.

In the present work we propose to answer two basic questions: firstly, whether the quantity of the SP-inactivating factor which, according to Lembeck (1956), is present in normal serum (NS) at a low level, increases during pregnancy (pregnant serum, PS), and secondly, whether this factor can be differentiated from diaminooxydase, oxytocinase and vasopressinase by specific inhibitors. The latter question is obviously very significant in judging the astructural relationship of SP and the posterior pituitary hormones. Moreover it is known that the enzymes specifically inactivating these hormones also occur at high levels in PS (Tuppy, 1960).

Method

The SP used had a potency of 6—8 U./mg. Testing was performed by the usual procedure using the isolated guinea pig intestine. The

enzyme preparation (PS) was made from retroplacental blood taken immediately after parturition and centrifuged at once. The PS used was never older than 24 hours. NS was obtained from healthy, non-pregnant women, and used within 24 hours. The following inhibitors have been used: aqueous aminoguanidine, 0.075 mg/ml, for diamino-oxydase (Schuler, 1952); p-chloromercuribenzoate (PCM), 0.38 mg/ml, for oxytocinase and vasopressinase (Werle, 1960) and the tripeptide S-benzyl-L-cysteinyl-L-leucyl-glycinamide (S-L), 0.0735 mg/ml, specifically for oxytocinase. We have included the examination of the effects of patulin, LSD, and SKF-525A on GS activity in view of the well known potentiation of the action of SP on isolated intestine by LSD (Werle and Effkemanon, 1940), and the inhibition of biogenic amines, H, 5-HT and ACh, but not SP, by patulin (Eliasson, 1958). Since we have found earlier that SKF-525A prolongs the action of SP (Stern and Dobrić, 1957), we also examined this compound.

1. series

5 mg SP was added to both 1 ml NS and 1 ml PS in order to see whether GS inactivates SP more strongly than NS.

2. series

Aminoguanidine was added to NS and PS to test whether the elimination of diaminooxydase influences the destruction of SP.

3. series

PCM was added to NS and PS in order to ascertain whether elimination of oxytocinase and vasopressinase influences the destruction of SP.

4. series

Aminoguanidine and PCM were added simultaneously to see whether inactivation of all three enzymes, diaminooxydase, oxytocinase and vasopressinase have any influence on the destruction of SP.

5. series

PS was treated with LSD (10^{-4} and 10^{-5} g/lit.)

6. series

PS was treated with patulin (10^{-4} and 15^{-5} g/lit.)

7. series

PS was treated with SKF-525A (10^{-5} g/lit.).

Results

Inactivation of SP by NS is weaker than that by PS (Fig. 1). PS is generally 5 to 6 times as active as NS, in some experiments even more than that. There is a distinct time-dependence; the decrease of contractions is obvious after 15, 30, and 60 min. incubation. Elimination of diaminooxydase has no effect upon the rate of SP inactivation (Fig. 2); it is to be recalled that PS is very rich in diaminooxydase (Werle, Effkemanon, 1940). Neither the elimination of oxytocinase by S-L, nor that of both, oxytocinase and vasopressinase by PCM influences the

FIG. 1

Contractions of guinea pig ileum produced by SP treated with normal serum (NS). SP was previously incubated with Tyrode's solution for 10 min. (control) (1), and with NS for 10, 30, and 60 min., respectively (2, 3, and 4).

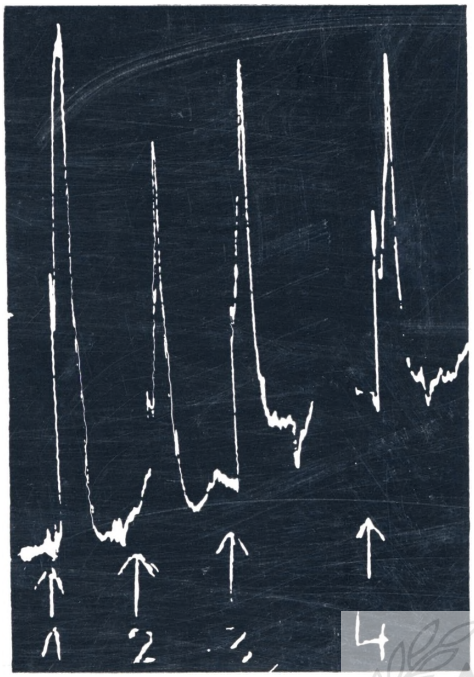


FIG. 2

Contractions of guinea pig ileum produced by SP treated with pregnant serum (PS). The experiment was performed as in previous Figure using PS instead of NS.



FIG. 3

Contractions of guinea pig ileum produced by SP and H treated with PS. Addition of SP, control (1), previously incubated with PS, alone (3), and PS in presence of aminoguanidine (5); addition of H treated in the same manner (2, 4, and 6).



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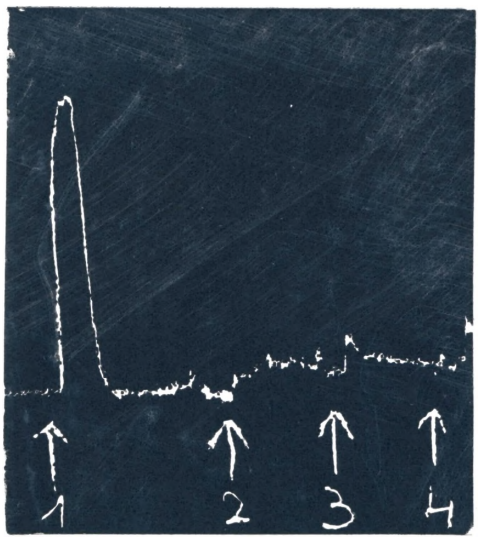


FIG. 4

Contractions of guinea pig ileum produced by SP treated with PS in presence of inhibitors. Addition of SP. control (1), previously incubated with PS, alone (2), in presence of PCM (3), and in presence of PCM and aminoguanidine (4).

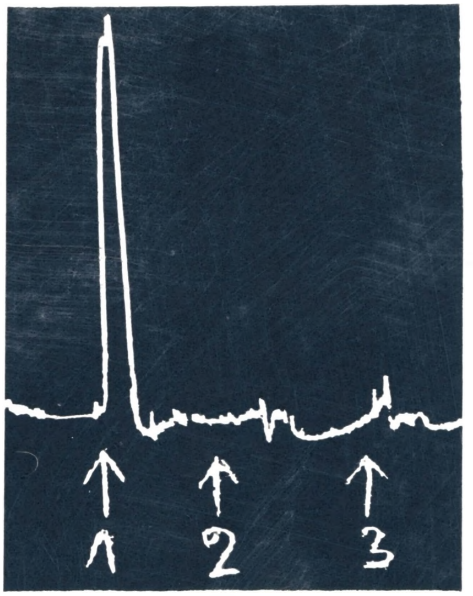


FIG. 5

Influence of patulin on inactivation of SP by PS. SP was previously incubated with Tyrode's solution for 10 min. (control) (1), and with PS in presence of patulin for 30 and 60 min., respectively (2, and 3).

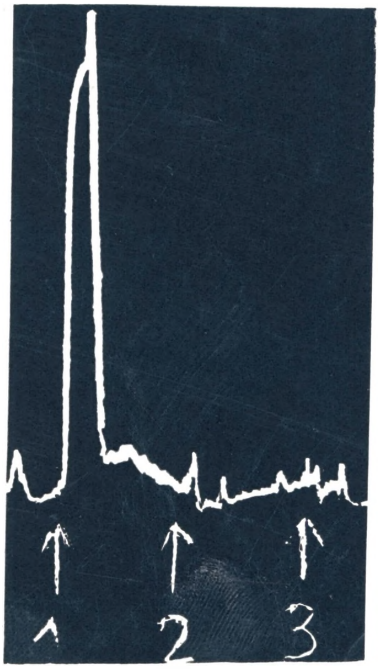


FIG. 6

Influence of SKF 525A on inactivation of SP by PS. Experimental conditions same as in previous Figure.

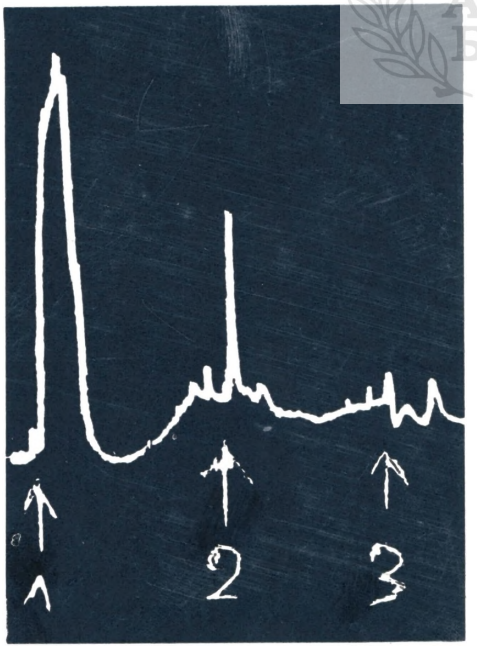


FIG. 7

Influence of LSD on inactivation of SP by PS. Experimental conditions same as in two previous Figures.

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destruction of SP by PS (Fig. 3). [It should be mentioned here that, according to Werle (1960), the vasopressinase activity of NS equals that of PS, whilst oxytocinase activity is markedly greater in PS.] The simultaneous elimination of diaminooxydase, oxytocinase and vasopressinase, likewise, has no effect on SP-ase activity (Fig. 4). Finally, as can be seen from Fig. 5, LSD, patulin and SKF-525A do not inhibit the inactivation of SP by PS. When LSD was added to PS prior to incubation with SP, there was still some SP activity left after 30 min., but after 60 min. (Fig. 6 and 7) complete inactivation occured. We are not able to tell at this moment whether this observation is due to a reduction of the rate of inactivation by LSD, or is it a consequence of the periphereal potentiation of the SP-activity in the isolated intestine by LSD as decribed by Krivoy (1957).

Discussion

It is to be concluded from these experiments that human serum contains an enzyme inactivating SP which is different from diaminooxydase, oxytocinase and vasopressinase. The enzymatic activity increases considerably during pregnancy. According to Matussek (1959) diaminooxydase isolated from the pea inactivates SP even in presence of aminoguanidine. We have obtained entirely different results with PS, which in presence of aminoguanidine inactivates SP, but not H. The difference between Matussek's findings and our own are probably due to the different sources of the SP-inactivating factor.

Arvidsson, Pernow and Swedin (1956), working with a diaminooxydase isolated from the kidney, were able to separate therefrom a factor inactivating SP. This speaks in favour of our own conclusions.

From our results it is impossible to tell what kind of enzyme performs the inactivation of SP. We only wish to stress that chymotrypsin destructs SP at a rate 200 times that of trypsin, a fact supporting the assumption about the polypeptidic character of SP. Krivoy (1957) observed that LSD inhibits a factor from guinea pig brain inactivating SP, but does not inhibit chymotrypsin. This finding was confirmed by Smith and Walaszek (1961). These authors found that the SP-inactivating factor from guinea pig ileum is likewise not inhibited by LSD, and that eserine potentiates the effect of SP on the gut. Since chymotrypsin is an esterase we could suppose that the SP-inactivating factor in PS is identical with chymotrypsin, and that in the guinea pig brain it is different from chymotrypsin. We would like to add that, according to Krivoy (1957), the guinea pig brain extract does not inactivate oxytocin, the same as we found for the factor from PS. The fact that patulin, acting spasmolytically by itself, and that SKF-525A do not influence the rate of inactivation of SP by PS, shows that SP-ase is not involved in the respective effects of these drugs on the action of SP. SKF-525A prolongs the tranquillizing effect of SP wich is a central one; we have not investigated the action of SKF-525A on the SP-inactivating factor in the brain. In connection with this problem it

is interesting to point out the results obtained by Kocić-Mitrović (1961), who found that the concentration of SP is lowered in the uterus and brain of pregnant rats, and the SP-ase activity in their serum is increased. Therefore the question arises whether SP plays some rôle in the mechanism of parturition having in mind that it produces contraction of smooth muscles in the uterus. Alternately, this rôle of SP could be deduced from the fact that increased SP-ase activity during pregnancy has the task of preventing contractions of the uterus by destructing excess SP, the same as it is the case with oxytocin and H.

We should mention the fact that already a few hours after parturition the SP-ase activity in the serum decreases. Even before parturition this activity is much less than in retroplacental serum.

Our results show that the serum of pregnant women contains a factor inactivating SP, which is not identical with diaminooxydase, oxytocinase and vasopressinase, and cannot be inhibited by LSD.

Summary

In retroplacental and pregnant serum there is an agent destroying SP. This agent is also found in the serum of normal women, but in much smaller quantities. The agent could not be inhibited by specific inhibitors of diaminoxydase, oxytocinase or vasopressinase. Pregnant serum incubated with SKF 525A enhanced the contractions of the isolated guinea pig ileum. Probably the agent is an enzyme similar, perhaps, to chymotrypsin.

FAKTOR SERUMA TRUDNICE KOJI INAKTIVIRA SUPSTANCIJU P

U retroplacentarnom serumu, a i u serumu gravidne žene, nalazi se neki agens koji razara SP. Ima ga i u normalnom serumu žene, ali u mnogo manjim količinama. Ovaj se faktor ne da inhibirati inhibitorima diaminooksidaze, oksitocinaze ili vazopresinaze. Retroplacentalni serum inkubiran sa SKF 525A pojačava kontrakcije izolovanog crijeva zamorca. Vjerojatno je da se radi o fermentu, koji je, možda, sličan kimotripsinu.

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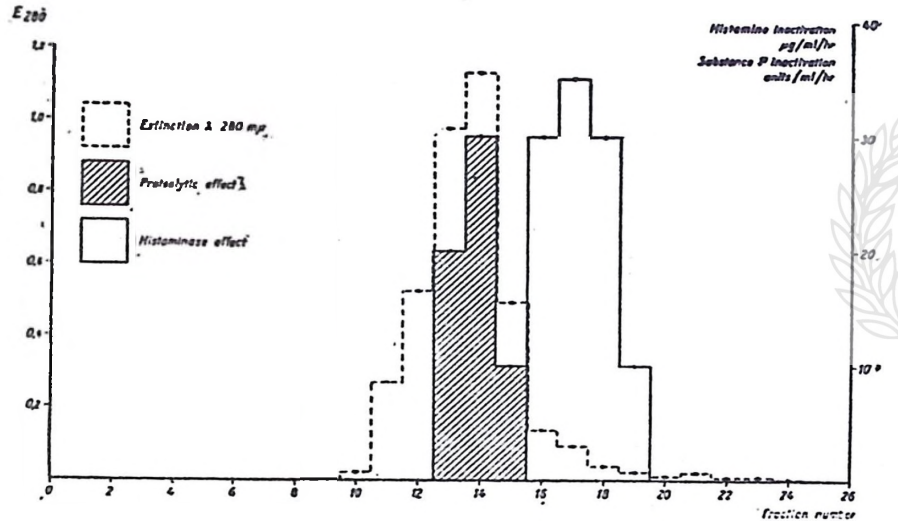
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DISCUSSION

PERNOW: As was mentioned in the paper, Arvidsson, Swedin an I (Acta physiol. scand. 1956, 35, 338) found that the proteolytic principle from kidney extracts, which inactivates SP was easily separated from the histaminolytic factor. The separation shown in the Figure was done on an ion exchange column (Dowex 50) using M/50 phosphate buffer, pH 7.1.



Separation of the histaminase and the substance P-inactivating agent in kidney extract on an ion exchange column (Dowex 50, 0.9 × 20 cm) in a M/150 phosphate buffer, pH 7.1. Effluent volume was 0.3 ml per 30 minutes.

Has anybody tried to extract SP from blood or urine?

STERN: Probably there is no SP in the blood. As far as I am aware there have been no attempts to extract SP from urine.

ZETLER: Matussek has found that diamine oxydase destroys SP. How do you explain the discrepancy to your findings?

STERN: Matussek extracted the enzyme from pea. We used the enzyme from human pregnant serum. We think that the different provenience of the enzymes might be the reason for this disagreement.

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