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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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