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STUDIES ON A PROTEIN-SYNTHESIS-AFFECTING SUBSTANCE FROM BIOLOGICAL MATERIALS

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SUMMARY

A substance of lipid character, containing carbohydrate and phosphorus, which stimulates the incorporation of labelled amino acids in the protein of Ehrlich ascites cells, was found in animal tissues and egg yolk. The content of the tissues of tumour-bearing individuals was higher than that of the tissues of normal ones. The substance was obtained in crystalline form.

INTRODUCTION

In a recent paper the effect was described of homogenates of various rat tissues on the incorporation of labelled methionine into the proteins of Ehrlich ascites cells *in vitro*[1]. It was found that these preparations exhibit a stimulatory as well as an inhibitory effect on this process, depending on the dose used. It seemed very probable that some substance present in the tissues might be responsible for these effects.

Some indications do already exist that there may be factors in tissues capable of influencing the biosynthesis of proteins[2]. Evidence was obtained[1] that tissues of tumour-bearing animals contain higher amounts of the active substance than those of normal ones. It seems quite reasonable to suppose that these different quantities of a protein-synthesis-affecting material in various tissues may be connected in some way with the varying abilities of these tissues to incorporate labelled amino acids *in vitro*. Such differences are known to exist between tumours and corresponding

normal tissues[3] as well as between similar tissues of tumour-bearing and normal animals[4].

It follows from our experiments that factors capable of stimulating the biosynthesis of proteins may possess a more general ability to enhance growth. A wide variety of growth-enhancing factors seems to exist in various tissues[5] and it is quite possible that some of them may exhibit this activity by influencing protein synthesis. This is true for the substance whose isolation will be described in this paper.

In our investigation attempts were made first to isolate the active substance from animal tissues. These attempts were successful but only a very small quantity of this material has been obtained in crystalline form. Egg yolks appeared to be a more suitable material for preparing it[6]. Yolks are known to contain a substance capable of inducing malignant growth[7] that seems to be identical with a growth-promoting factor described by other authors[8]. It seemed very probable to us that this substance may be also identical with that responsible for the stimulation of protein synthesis[1] and enhanced growth of malignant tissues[9]. This possibility was strengthened by the fact that carcinogenic substances seem to affect the protein synthesis in the same dose-dependent way as did tissue homogenates[10].

MATERIALS AND METHODS

Biological materials

Wistar rats of both sexes, weighing 150–200 g, were used. They were fed on a Larsen diet and given water *ad libitum*. Walker 256 carcinoma was implanted subcutaneously. Two weeks after the transplantation of the tumour the animals were killed by a blow on the head and the tissues immediately excised. For preliminary experiments, lyophilized liver tissue from normal rats was used. A 10 % homogenate in water was freeze-dried by a method described elswhere[11]. The lyophilized tissue was stored in a deep-freezing unit (—30°) and used after 7 months of storage.

Mice, from which tissues for some preparations originated, were of a mixed albino stock, weighing 20–25 g, of both sexes. They were maintained on Larsen diet and given water *ad libitum*. Animals bearing subcutaneous grafts of Sarcoma 37 were used 2 weeks after implantation.

The Ehrlich ascites carcinoma, used for the study of incorporation of labelled amino acids into its proteins, was a frozen preparation as described in our previous paper[1].

Fresh fertilized eggs from white Leghorn hens were used as starting material for preparation of the active substance from yolks.

Chemicals

All chemicals used in our experiments were of A.R. grade. All solvents were redistilled before use. The petroleum ether used was a fraction boiling between 60 and 80°. Alumina used for column chromatography was standardized according to BROCKMANN[12]. To obtain the highest activity grade, alumina was heated for at least 5 h at 300° in an iron dish and stored immediately under petroleum ether. All preparations of alumina were tested for their activities according to BROCKMANN[12].

Labelled amino acids used in our experiments were ^{35}S-labelled *dl*-methionine, with an average activity of 0.6–0.7 mC/mg, and ^{14}C-carboxyl-labelled glycine, with

an average activity of 0.16 mC/mg. Both these compounds were obtained from the Radiological Research Institute in Prague. A solution of 1.5 mg methionine in 100 ml of an isotonic $KCl-KHCO_3$ mixture (1000:8) and 26 mg glycine in 10 ml of the same solution was used.

Chemical methods

Alumina chromatography was performed under atmospheric pressure in glass tubes of varying diameters and lengths provided with a stopcock.

Paper chromatography was performed using the descending technique. The following solvent systems were used: (1) water-saturated n-butanol, Whatman No. 1 paper, (2) water-saturated collidine, Whatman No. 1 paper, (3) chloroform–methanol (1:9), Whatman No. 3 paper, (4) acetic acid–chloroform–liquid paraffin (65:25:10), Whatman No. 3 paper impregnated with 10 % liquid paraffin in petroleum ether, (5) methanol–n-butanol–water (80:5:15), Whatman No. 3 paper impregnated with 10 % liquid paraffin in petroleum ether, (6) benzylalcohol, Whatman No. 3 paper impregnated with 20 % liquid paraffin in petroleum ether.

For the detection of various spots, the fluorescence of the dried chromatograms was first examined using a high pressure mercury vapour-lamp (Philips Philora HPW 125) equipped with a Wood glass filter. For the detection of steroids, antimony trichloride was generally used[13]. The dried chromatograms were immersed in a saturated solution of antimony trichloride in chloroform and dried for 5 min at 120°. For a more sensitive detection, chromatograms treated in this way were examined using an ultraviolet lamp as mentioned above. Occasionally iodine[14], phosphowolframic[15] and phosphomolybdenic acid[16], and blue tetrazolium[17] were used for the detection of spots.

For the detection of carbohydrates, spraying of the chromatograms with the benzidine reagent[18] was the method most used. Aniline hydrophthalate[19] and naphthoresorcine[20] were used in some instances.

Determination of nitrogen was carried out by MARKHAM's modification of the Kjeldahl method[21], determination of sugars by the orcinol method[22], using equimolar glucose and mannose mixture as a reference solution. Phosphorus was determined by the method of FISKE AND SUBBAROW[23] and cholesterol on the basis of the Liebermann–Burchhardt reaction[24]. Determinations of optical densities were performed in all instances in a Lange photoelectric colorimeter, using monochromatic metal interference filters.

Assay of biological activity

This was made by studying the effect of various preparations on the incorporation of labelled amino acids into the proteins of Ehrlich ascites carcinoma. All fractions were tested in triplicate. Each reaction mixture in a conventional Warburg flask contained 2 ml of Ehrlich ascites tumour suspension (diluted to contain 0.5 to 0.7 % protein with an isotonic $KCl-KHCO_3$ mixture), 0.1 ml of the labelled amino acid solution, and 0.1 ml of the appropriate dilution of the fraction to be tested or of the blank isotonic $KCl-KHCO_3$ mixture. 9 different doses of each preparation, varying from 1 mg to 10^{-8} mg, were usually tested for their effect on incorporation. In each run the control samples were incubated simultaneously with the experimental ones by shaking them in a Warburg apparatus at 37.5° for 45 min.

The method of preparing the protein samples after incubation as well as that used for the measurement of their specific radioactivity is given in detail in our recent paper[1].

Zero-time protein samples were always prepared in duplicate. The counts/min value of these zero-time samples did not exceed 10 % in the case of methionine and 4 % in the case of glycine, of the counts/min values of the incubated samples (these being usually 190–220 counts/min/mg of dried protein precipitate in the case of methionine, and 30–40 counts/min/mg of protein, in the case of glycine). Standard errors did not exceed $\pm$ 3 % for duplicate protein samples from each flask, and $\pm$ 5 % for average duplicate values of three identical reaction mixtures.

For the evaluation of activity of the various preparations, the mean specific radioactivity of protein sample was divided by that of a control protein sample obtained from a reaction mixture containing no tissue preparation (control). Only preparations, of which at least two neighbouring doses showed a definite stimulatory effect (at least 120 % of the control specimen's value) were said to be active. Fractions showing only a slightly inhibitory activity in the highest doses used or no effect on the incorporation at all dose levels were said to be ineffective.

EXPERIMENTAL

Preliminary fractionation of rat liver tissue

The active substance appeared to be rather stable. Freeze-dried liver retained its activity for several months. The activity of homogenates prepared from it did not diminish for at least 5 days at 20°. However, repeated slow freezing and thawing of the homogenate completely destroyed its activity. Freeze-drying left the activity unimpaired.

The active substance appeared to be present in the supernatant of a liver homogenate centrifuged at 20,000 *g* for 20 min at 18°. It did not dialyse and was not destroyed by trypsin. The activity was not affected by heating a homogenate at 70° for 60 min. On the basis of these facts, in particular the maintenance of the original activity after digestion or heating, it seemed very improbable that a protein would be responsible for the effect. On the other hand, the fact that the active principle became dialysable upon incubation with trypsin suggested that a low-molecular substance attached to a protein carrier might be involved in the effect. Therefore the possible involvement of protein-bound lipid was investigated.

For this purpose, freeze-dried rat liver was extracted in a Soxhlet apparatus with various solvents. In each case, 1 g of material was extracted with 200 ml of the solvent for a period sufficient to obtain an colourless fluid in the last portions of the extract. The reddish-brown total extract was evaporated to dryness under reduced pressure below 50°, the residue was homogenized in a glass homogenizer in isotonic $KCl-KHCO_3$ solution, and the resulting suspension, made up to the volume of a 1:10 homogenate of a corresponding amount of liver, was tested.

Benzene extract, ether extract, and acetone extract showed the same enhancing effect as the original homogenate. However, the ethanol extract was inactive. These results favoured our assumption that some lipid substance might be responsible for the activity.

Subfractionation of the unsaponifiable lipid fraction from varous tissues

It was found in some preliminary experiments that tissues (not only the tumours) from tumour-bearing animals seemed to contain a much higher quantity of the active substance than do the corresponding organs of normal animals. Therefore Walker tumour tissue and livers from rats bearing this tumour, as well as Sarcoma 37 tissue and livers from mice bearing this tumour, were used in these fractionation experiments.

In each case, tissues were homogenized in ethanol in a blendor and refluxed with ethanolic potassium hydroxide. The resulting suspension was extracted with petroleum ether and this extract was then evaporated to dryness under reduced pressure. The dry residue was dissolved in petroleum ether and filtered. The solution obtained was sucked through an alumina column (Grade I). The column was eluted by a series of solvents and solvent mixtures (see Table I) of increasing polarity.

TABLE I

COMPOSITION OF VARIOUS SOLVENT MIXTURES USED FOR ELUTION OF ALUMINA COLUMNS

Composition	*Fraction No.*
Petroleum ether	1
Benzene (25 %) in petroleum ether	2
Benzene (50 %) in petroleum ether	3
Benzene (75 %) in petroleum ether	4
Benzene	5
Chloroform (25 %) in benzene	6
Chloroform (50 %) in benzene	7
Chloroform (75 %) in benzene	8
Chloroform	9
Acetone (25 %) in chloroform	10
Acetone (50 %) in chloroform	11
Acetone (75 %) in chloroform	12
Acetone	13
Ether (25 %) in acetone	14
Ether (50 %) in acetone	15
Ether (75 %) in acetone	16
Ether	17
Ethanol (25 %) in ether	18
Ethanol (50 %) in ether	19
Ethanol (75 %) in ether	20
Ethanol	21
Ethanol (75 %) in water	22
Ethanol (50 %) in water	23
Ethanol (25 %) in water	24
Water	25

In a typical experiment of this kind, 1000 g (wet weight) liver from Walker tumour rats was homogenized in 2000 ml 96 % ethanol in a blendor. 100 g potassium hydroxide were added to the homogenate, which was then refluxed for 4 h on a boiling water bath. After cooling and adding 200 ml water, extraction was carried out by shaking 10 times with a total volume of 30 l petroleum ether. The resulting brilliant yellow solution was evaporated to dryness under reduced pressure below 35°. 15 g of a reddish-brown dry residue was obtained. This was shaken with 500 ml petroleum ether, the resulting somewhat turbid solution filtered, and the clear, bluish-violet fluorescing filtrate poured on a column of 150 g alumina, activity I (diameter 25 mm,

length 820 mm). After the solution had passed the column, the latter was successively washed with 500 ml each of the solvent mixtures given in Table I. After the last drop of a particular fluid had entered the top of the column, 500 ml of the next fluid was applied and a new fraction collected. Each fraction was evaporated to dryness under reduced pressure below 35°, the residue dissolved in a small volume of an appropriate solvent and tested by paper chromatography (Whatman paper No. 3, acetic acid–chloroform–liquid paraffin). Fractions giving corresponding chromatographic patterns were pooled. In this way the fractions designed by 605 (pooled fractions 1–5), 610 (6–10), 624 (11–24), and 625 (25) were obtained. Their chromatographic behaviour is illustrated by Fig. 2. These pooled fractions were evaporated to dryness, the residue of each was homogenized in isotonic KCl–$KHCO_3$ buffer in a glass homogenizer to give a 0.1 % suspension. These suspensions were tested.

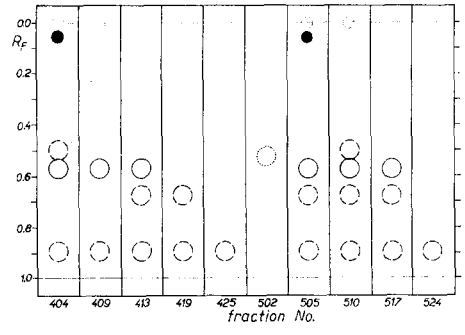

Fig. 1. Chromatographic pattern of unsaponifiable lipids of mice liver (fractions No. 404–425) and Sarcoma 37 tissue (fractions No. 502–524) in acetic acid–chloroform–liquid paraffin mixture. ●, Substances giving both steroid and sugar reactions; ———, steroid reactions only; – – – –, sugar reactions only; ·····, compounds giving blue fluorescing spots on unsprayed paper and no other reactions.

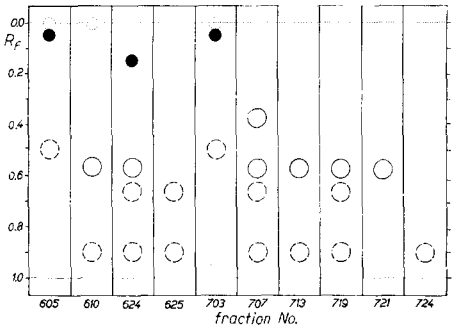

Fig. 2. Chromatographic pattern of unsaponifiable lipids of rat liver (fractions No. 605–625) and Walker tumour tissue (fractions No. 703–724) in the same solvent mixture as in Fig. 1.

TABLE II

EFFECT OF FRACTIONS PREPARED FROM VARIOUS ANIMAL TISSUES ON THE INCORPORATION OF LABELLED METHIONINE INTO THE PROTEINS OF EHRLICH TUMOUR *in vitro*

*Fraction No.**	*Dose (10^{-n} mg)*									*Activity*
	0	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	
605	123	62	70	96	75	80	132	220	215	+
610	69	182	252	99	96	63	132	110	137	+
624	117	169	152	75	60	95	153	123	112	+
625	85	93	87	59	85	78	71	74	70	—
703	65	128	79	131	91	108	72	97	102	+
707	68	97	72	79	69	81	90	68	59	—
713	96	54	81	228	163	190	139	134	128	+
719	63	83	90	69	84	76	129	125	92	+
721	34	38	116	92	49	128	114	82	83	+
724	89	89	95	123	132	110	93	104	101	+

All values given in this table are in percentage of control specimen's value.

* Fractions No. 605–625 are prepared from livers of tumour-bearing rats, fractions No. 703–724 from the Walker tumour tissue. The two last numbers denote the number of the pooled fraction (for instance fraction 605 = pooled fractions No. 1–5, 610 = pooled fractions No. 6–10, etc.).

In the corresponding fractionation of the petroleum ether extracts from ethanol–KOH digests of Walker tumours (680 g), Sarcoma 37 of mice (35 g), and liver of mice bearing this tumour (55 g), the volumes of elution fluids used were proportional to the amount of tissue.

Chromatographic patterns of various fractions from these tissues are given in Figs. 1 and 2, and their effect on the incorporation in Table II. Obviously most fractions are active.

Only in the case of rat liver tissue was it possible to recover a few milligrams of a crystalline active substance. For example, a portion of the residue of fraction 605 was dissolved in a small volume of ether and ethanol was added until a slight opalescence appeared. After a few days in an ice-box fine needle-shaped crystals appeared, which after two recrystallizations possessed a constant melting point of 65° and showed a chromatographic behaviour identical with that of the substance isolated from egg yolks described below. This crystalline product was active.

In all other instances identical material was found on chromatograms but the amount of substance scarcely sufficed to give distinct spots.

Fractionation of the active principle in the unsaponifiable portion of hen's egg yolks

For reasons mentioned in the introduction, it was decided to test egg yolks, which seemed a promising source of crystalline material. The fractionation methods used for animal tissues were followed as closely as possible in these experiments.

Yolks from very fresh eggs were homogenized in a blendor and refluxed with ethanolic potassium hydroxide. The resulting suspension was reduced to a small volume and extracted by benzene. The residue after extraction with benzene was tested for its activity in the usual way. It was found to be inactive, essentially all active material being present in the benzene layer. The benzene solution was evaporated and the residue dissolved in a small volume of petroleum ether. This was then poured on an alumina column.

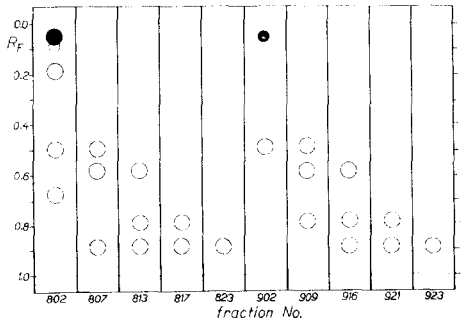

Fig. 3. Chromatographic pattern of unsaponifiable lipids of egg yolks fractionated on columns of inactive (fractions No. 802–823) and active alumina (fractions No. 902–923). The same solvent mixture was used as in Fig. 1.

Since preliminary experiments had suggested that some destruction of the active substance might occur on columns of highly active alumina, two columns, one of highly active grade I (the same as in fractionations of animal tissues) and another of weakly active grade IV alumina, were used in this experiment.

The petroleum ether solution obtained from 50 yolks was divided into two equal parts and poured on the two columns of alumina (diameter 25 mm, length 550 mm). They were then eluted with solvent mixtures as given in Table I (25 ml each). Again the chromatographically identical fractions were pooled and their activity tested. Chromatograms of these pooled fractions are given in Fig. 3.

Obviously, only small chromatographic dissimilarities exist between the fractions obtained from the two columns, while the similarity with the products from animal tissues is striking. On the other hand, very distinct differences do exist between the fractions obtained with both types of alumina as far as their effect on the incorporation is concerned (see Table III). While essentially all fractions of the grade I alumina column were active, only the first and the last fraction from the grade IV alumina proved to be effective. It seems very probable that a decomposition takes place on active alumina yielding substances, which are still active in amino acid incorporation.

TABLE III

EFFECT OF VARIOUS FRACTIONS PREPARED FROM EGG YOLKS ON ACTIVE AND INACTIVE COLUMNS OF ALUMINA ON THE INCORPORATION OF LABELLED METHIONINE INTO THE PROTEINS OF EHRLICH TUMOUR

*Fraction No.**	*Dose (10^{-n} mg)*									*Activity*
	0	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	
802	117	125	130	118	75	71	73	78	82	+
807	86	91	96	99	101	101	101	93	101	—
813	75	70	72	93	90	86	94	102	95	—
817	91	86	72	78	89	88	91	86	72	—
823	83	74	92	91	141	130	102	70	89	+
902	106	100	115	129	158	131	69	79	83	+
909	128	107	105	56	81	61	123	183	145	+
916	35	73	73	95	62	78	86	105	101	—
921	105	90	123	83	87	85	105	139	218	+
923	101	100	118	129	136	115	110	102	104	+

All values given in this table are in percentage of control specimen's value.

* Fractions No. 802–823 = inactive alumina (grade IV), fractions No. 902–923 = active alumina (grade I). Composition of each fraction is indicated by both last numbers as in Table II.

In an attempt to isolate the active substance in crystalline form, small portions of residues of fractions 802 and 902, respectively, were dissolved in ether, and ethanol was added until the solution became slightly opalescent. After a few hours in the ice-box a heavy crop of very fine needle-shaped crystals appeared. This crystalline substance had an identical melting point of 65° and an identical chromatographic behaviour in various systems as the crystalline substance isolated from rat liver.

Method of preparation of the active crystalline substance from egg yolks

The yolks from 50 fresh eggs were homogenized in a blendor and refluxed with 1000 ml 10 % ethanolic potassium hydroxide for 4 h. After cooling, the fine greenish-brown suspension was concentrated at 40° under reduced pressure to a viscous dark green liquid (400 ml). This was extracted in several small portions by shaking with benzene. It is necessary to use a large excess of benzene (10–15 times the volume of the concentrated ethanol–KOH digest in each run) to obtain a good separation of

the layers. The extraction was continued until the benzene layer remained uncoloured. Opalescent yellow benzene extracts (28 l) were pooled, centrifuged at 30,000 *g* in a Sharples centrifuge and the resulting brilliant yellow solution evaporated at 50° under reduced pressure. The dry, reddish-brown residue was dissolved in 400 ml 30% benzene in petroleum ether. The turbid dark brown solution was filtered and the resulting clear filtrate poured on a column prepared from 1000 g of alumina, grade IV (diameter 50 mm, length 460 mm). The column was eluted with 11 l 30% benzene in petroleum ether until the effluent solution gave no more brilliant bluish-violet fluorescence in ultraviolet light.

The eluate was evaporated at 40° under reduced pressure. A brown, oily residue was obtained, which solidified on standing at room temperature. This was dissolved in a small volume of ether, after which ethanol was added until the resulting solution appeared slightly opalescent. During the next 5 min the first very fine needle-shaped crystals appeared. Crystallization was completed at —30° overnight (lower temperatures were found preferable for higher yields of crystals). The crystals were spun down at 0° and washed several times with ethanol. The mother liquids were concentrated, and a second and a third crop of crystals obtained on standing at —30°. The crystals were pooled and after drying for 4 days in a high vacuum (0.05 mm Hg) 15 mg of a greasy, colourless substance was obtained.

After three recrystallizations from ethanol–ether the melting point was constant at 65°. The substance gave only one spot when chromatographed in five various solvent systems.

Some chemical properties of the crystalline substance

Elementary analyses showed the presence of carbon, hydrogen, oxygen, and phosphorus. At room temperature the substance is very soluble in petroleum ether, benzene, toluene, ether, chloroform, propanol, butanol, and cyclohexanol, but only very sparingly in acetone. It is also sparingly soluble in hot methanol and ethanol, and insoluble in cold or boiling water.

It gives colour reactions typical for steroids[25] (Liebermann–Burchhardt, Rosenheim, and Salkowski tests). It is not precipitated by digitonine. On paper chromatograms, it can be detected by iodine, phosphowolframic and phosphomolybdenic acid, blue tetrazolium, aniline hydrophthalate, naphthoresorcine, and in particular with benzidine. On papers sprayed with 10% trichloracetic acid and heated at 100° [26] it gives an intensively blue fluorescing spot. It also gives a blue fluorescence on unsprayed paper, but this is rather weak. It readily reacts with antimony trichloride to give an intensive reddish-pink spot on the paper.

In solution the substance gives some positive carbohydrate reactions, *e.g.* a deep red colour upon heating with phloroglucine, while the reactions with naphthoresorcine and orcine are also positive.

Paper chromatography is most conveniently carried out with solvent system (2). In water-saturated butanol the R_F value is extremely high (0.93), in acetic acid–chloroform–liquid paraffin, on the other hand, unsatisfactorily low (0.05). In the further systems tested, the active substance appeared not to move from the starting point at all. With the exception of water-saturated butanol all solvent systems mentioned on page 34 proved to be very efficient for detecting impurities in the

crystalline preparations. For this reason they were all applied in our routine test for checking the purity of preparations.

The crystalline substance seems to be rather unstable. Upon prolonged heating or storing in solution for a long period of time it appears to decompose. It was found that its extinction coefficient at various wavelengths in the ultraviolet region is rapidly changing. It seems that, in particular, light and atmospheric oxygen are harmful. For this reason several steps of the isolation procedure, especially the crystallization, are now being performed under nitrogen. Upon standing in crystalline form the substance becomes yellowish brown and various products are formed, which can be distinguished from the original substance by paper chromatography.

The activity of the crystalline substance

Fig. 4 illustrates the enhancement as well as the inhibition of the incorporation of glycine and methionine in ascites protein by various doses of the substance. It is difficult to understand why two zones of inhibition are separated by a zone of activity. In particular the inhibition at low concentrations is obscure. It may be that the substance still contains an inhibitory factor whose action cannot be detected at higher concentrations of the stimulation factor, the latter also becoming inhibitory at still higher concentrations.

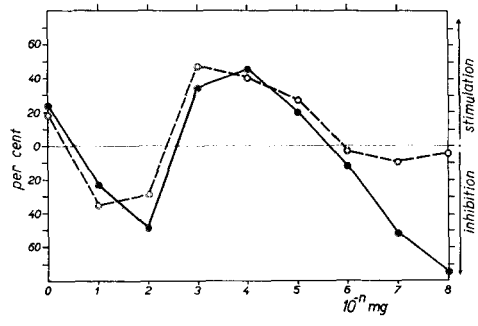

Fig. 4. Effect of the crystalline compound on the incorporation of labelled glycine (———) and methionine (– – – –) into the proteins of Ehrlich tumour *in vitro*. Individual points indicate averages of 5 experiments carried out with the same batch of Ehrlich ascites. Results of individual experiments did not differ more than ± 8%. Statistical treatment (*t*-test) of most values gave $P < 0.001$ when compared with the control.

It was shown above (see Table III) that some fractions isolated from yolks as well as animal tissues on active alumina have only the enhancing effect.

The inhibitory compound may be regarded as a destruction product of the active substance since similar compounds were also found in solutions of crystalline substance left for longer periods of time.

It is also possible that one substance is capable of influencing various enzymes resulting in various overall effects of different doses.

The values given in Fig. 4 are the results of experiments with one particular batch of Ehrlich ascites. With different batches, different absolute values for stimulation or inhibition were obtained. Nevertheless, the maximum stimulatory effect was always found at exactly the same dose. The stimulatory effect of the highest dose was always present; in some batches the 0.1 mg dose also had an enhancing effect.

DISCUSSION

The same substance seems to be present in such widely different materials as animal tissues and egg yolks. Further experiments[27] showed that it is capable of stimulating the growth of young animals very considerably when added to their diet. Therefore its presence as a growth-stimulating factor in relatively high amounts in egg yolks is understandable. The active substance was isolated only from fresh fertilized eggs. We are not yet quite sure if it is necessary for the eggs to have been fertilized. It is essential, however, that very fresh eggs are used.

The higher quantity of the active substance present in tissues of tumour-bearing animals as compared with those of normal ones suggests the relation of this compound to malignant growth.

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