stimulation at a series of points along the floor of the hypothalamus and telencephalon. Twelve points were selected, in 12 different animals. After implantation and a 14-day recovery period, animals were trained to press a lever for chemical stimulation with iproniazid; in this case there was no prior training with electric stimulation.

Whereas all animals with ventral hypothalamic pipettes showed a better-than-chance tendency to press the bar for injection of the chemical, there was a distinct differentiation of rate of self-injection, depending on placement of the pipette. Pipettes in the posterior hypothalamus gave higher rates of self-injection than pipettes in the anterior hypothalamus. And high rates of self-injection were also obtained with pipettes in the dorsal preoptic region. These differences agree well with differences with respect to brain area in rate of self-stimulation obtained in earlier experiments with electric stimulation (*5*).

When the injector was turned off, so that bar-pressing no longer produced self-injection, animals continued to press the bar for some time (see Fig. 1), as though no change had been made. This is probably attributable to the high level of the chemical in the brain at these points and to the gradual working down of residual stores in the pipette. Extinction does eventually result from termination of the flow, after a period of about 30 to 45 minutes. Also, when the animal is shifted from an extinction period to a new self-injection period, rate of responding quickly changes from chance levels to rates of about 300 an hour. Thus, it is the chemical reward which sustains the behavior.

Injection of serotonin, by itself or immediately after injections of iproniazid, caused the animals to lie down. An animal lying down after injection of serotonin could be brought back to its feet by epinephrine. This, taken together with the tendency of some animals to press the bar for epinephrine, suggests that the exciting and rewarding effects of iproniazid are connected more with epinephrine than with serotonin.

From the experiments on self-injection, three main results have been gained. Substantively, we have learned that iproniazid is an excitant of reward functions in this motivational system of the hypothalamus, and that quite probably it has this excitatory function in common with epinephrine. Methodologically, we have validated a technique of self-injection which can now be used to resolve further the problem of the excitatory and inhibitory chemistry of the motivational systems. Finally, from the clinical point of view, we have been exceptionally fortunate in having our method select the pharmacological agent which has recently given best results in alleviating depressions, for this indicates that it is a method for locating antidepressants.

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30 December 1957

A New Phospholipid, Malignolipin, in Human Malignant Tumors

We report here the discovery of a new phospholipid—malignolipin—containing spermine, found specifically in malignant tumors, but never in normal tissues. Laborious efforts of many of our predecessors have been concentrated on the problem of finding a substance which exists only in malignant tumors and never in normal tissues, but such efforts have been unsuccessful up to the present.

In examining the affinity of various cell components for porphyrin (*1*), we noticed the very marked affinity of extracellular small bodies in cancer tissues for protoporphyrin III (*2*), and we also ascertained that the substance in normal tissues which has an affinity for protoporphyrin III, and which occurs in mitochondria and myelin sheaths, is sphingomyelin (*3*). Investigation of the chemical nature of the extracellular small bodies in cancer tissues with the marked affinity for protoporphyrin III has led to the discovery of a new phospholipid.

Sphingomyelin can be extracted by means of boiling 95-percent ethanol; the substance other than sphingomyelin with affinity for protoporphyrin III is isolated from freshly excised human malignant tumors (a seminoma, a stomach cancer, a colon cancer, a uterine cancer, a breast cancer, and Hodgkin's malignant granuloma were used in the studies reported here) by fractionation with organic solvents, as follows. A freshly excised malignant tumor is extracted with 9 volumes of boiling ethanol and filtered while hot. The filtrate is left at 0°C overnight, and the supernatant is evaporated to dryness in a vacuum, then extracted with absolute ethanol. The supernatant is added, with 2 volumes of acetone, and the solution is left at 0°C overnight. The precipitate is washed with acetone-ethanol (2:1) and then with acetone; then it is dried in a vacuum and dissolved in ether. The supernatant is added, with 2 volumes of acetone, and the solution is left at 0°C overnight. The precipitate is washed with acetone, dried in a vacuum, and dissolved in absolute ethanol. The supernatant is added, with 2 volumes of acetone, and the solution is left at 0°C overnight. Such precipitation with acetone from ether and ethanol solution is repeated till the final precipitate contains no trace of the substance that has no affinity for porphyrin (*3*). The precipitate is washed with acetone-ethanol (2:1), then with acetone; then it is dried in a vacuum and dissolved in chloroform. The supernatant is added, with 2 volumes of acetone, and the solution is left at 0°C overnight. The precipitate is washed with acetone, freed from acetone, and crystallized from chloroform. The substance with the affinity for protoporphyrin III was obtained thus, in pure state, as small hexagonal, snow-crystal-like crystals, from every one of the aforementioned malignant tumors.

This compound is very hygroscopic, is strongly basic, and is readily soluble in water, ethanol, ether, petroleum ether, and chloroform but is insoluble in acetone. In every instance, the compound obtained from the aforementioned tumors was found to contain nitrogen and phosphorus in the ratio of 5 to 1 and, quite unlike all other phospholipids that have ever been reported, to show only one spot; this spot can be revealed by Ninhydrin, as well as by Dragendorf's reagent, on the paper chromatogram developed with *n*-butanol, butanol–acetic-acid–water (4:1:5), or ethanol-water (8:1). Moreover, the compound was found not to be contaminated with other substances (the biuret test, Ehrlich's aldehyde test, Molisch's test, Bial's test, Pettenkofer's test, Feulgen's test, and the Liebermann-Burchard test were all negative).

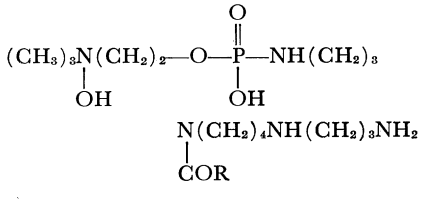
When this lipid is left in 0.3*N* HCl at 18°C for 3 hours, the opalescent solution becomes quite transparent, with some oily droplets at the surface, the whole amount of phosphorus contained in the original lipid can be recovered as free phosphoric acid, and no trace of acetone-insoluble original lipid can be obtained from its ether extract—that is to say, the lipid is completely hydrolyzed.

The ether-soluble part of the hydrolyzate contains neither phosphorus nor nitrogen, and the following tests have all been negative: Bial's test, Molisch's test, Ninhydrin test, sodium fluorescein test, Feulgen's test, and the Liebermann-Burchard test. It has been ascertained that the ether-soluble part of the hydrol-

yzate is composed of some unsaturated fatty acid, which is solid at a temperature lower than 60°C and is obtained in rhombic crystals from ethanol solution.

The ether-insoluble part of the hydrolyzate is found to contain the whole amount of nitrogen and phosphorus involved in the original lipid and to contain no trace of Bial-positive or Molisch-positive substance. On paper chromatography of desalted hydrolyzate with butanol–acetic-acid–water (4:1:2), only one spot can be detected by means of a Dragendorf's reagent; this appears at a place quite similar to that of choline hydrochloride. Beautiful crystals of choline reineckate can also be obtained from the hydrolyzate. On paper chromatography of the desalted hydrolyzate with butanol–acetic-acid–water (4:1:5), only one nice purple spot can be detected by means of a Ninhydrin spray, at a place quite similar to that of spermine hydrochloride isolated from pigs' semen; no other Ninhydrin-detectable spot was ascertained. Choline in the hydrolyzate is, then, precipitated quantitatively as reineckate, and the optical density of an acetone solution of the reineckate at 327 mμ is measured spectrophotometrically. Spermine in the hydrolyzate is precipitated with phosphotungstic acid, the precipitate is extracted with chloroform after digestion with 50-percent K_2CO_3, and the nitrogen in the fraction extracted with chloroform is measured; this must be the nitrogen of spermine. The sum of the choline nitrogen and spermine nitrogen agrees well with the total nitrogen content of the hydrolyzate. It has also been demonstrated that phosphoric acid, choline, and spermine in the hydrolyzate are equimolar.

The existence in human malignant tumors of a phospholipid which has a marked affinity for protoporphyrin III and which is composed of choline, spermine, phosphoric acid, and fatty acid (as is shown by the following tentative formula) is thus confirmed, though the arrangement of the components and the number and kinds of fatty acid are yet to be revealed. We propose to designate this lipid "malignolipin." The tentative formula is



It has been ascertained that malignolipin is never found in normal tissues, such as cattle brain or whole bodies of normal mice.

As malignolipin is found to exist richly in tumors of high malignancy and in the rapidly growing part of a tumor and scantily in necrotic tumors or in the degrading part of a tumor, this lipid is supposed to be intimately related to the malignancy of tumor cells.

The discovery of a new phospholipid, which is found only in malignant tumors and never in normal tissues, will greatly contribute not only to the diagnosis of malignant tumors but also to the elucidation of their pathogenesis and, further, to the discovery of means to make them subside.

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17 December 1957

Free Radical Formation in Reaction between Old Yellow Enzyme and Reduced Triphosphopyridine Nucleotide

In 1937 Haas (*1*) described the transitory appearance of a red color when old yellow enzyme was reduced in the presence of an excess of triphosphopyridine nucleotide (TPN). The absorption maximum of the free enzyme at 465 mμ was shifted to 475 mμ. Haas considered the red complex to be a free radical, and others have cited this as probably representing the earliest evidence for free radical intermediates in oxidation-reduction enzymes (*2*). More recently, Beinert has described spectroscopic changes accompanying oxidation-reduction of flavin mononucleotide (FMN) (*3*) and a number of flavin-containing enzymes (*4, 5*) which he attributed to free radical formation. These consisted, chiefly, in the transient appearance of a broad absorption band with maximum at 565 mμ. These spectral changes were least conspicuous in the case of old yellow enzyme. Nevertheless, Beinert was unable to confirm Haas's observation regarding the formation of a red complex. The work described in this report was undertaken to elucidate further the reaction between old yellow enzyme and reduced TPN (TPNH) in an attempt to resolve the differences between the findings of Beinert and Haas and to prove whether or not a free radical is actually formed.

Old yellow enzyme of high purity was prepared according to the method of Theorell and Akeson (*6*). Absorption spectra in clear solutions were studied with a Beckman DU spectrophotometer; microcells of 0.3 ml volume and 1 cm light path were used. Oxygen could be excluded by displacement with nitrogen and by stoppering the cuvette.

In a number of experiments under a variety of conditions, we have been able to confirm Haas's observation on the formation of an orange-red compound. An essential requirement appears to be the presence of the pyridine nucleotide in the reduced form, but addition of hydrosulfite or exclusion of oxygen is not necessary.

The red compound is best obtained by addition of a 10- to 15-fold excess of TPNH to a solution of old yellow enzyme in neutral phosphate buffer. The entire absorption spectrum is shifted about 10 mμ toward longer wavelengths as compared with that of old yellow enzyme (Fig. 1). The shift of the maximum absorption peak is obscured when a steep background absorption is present, as in the case of Beinert's experiments (*5*). At least a part of the small absorption increase between 550 and 650 mμ that he observed must have been due to this shift in absorption peak. In spite of several attempts, under varied conditions, we have been unable to observe any distinct peak in that spectral region.

By ultracentrifugation in a separation cell of the reaction mixture containing the red complex, it was established that this is a compound between old yellow enzyme and TPN. Spectrophotometric assay of the pyridine nucleotide remaining uncombined in the supernatant fraction indicated that the amount of pyridine nucleotide bound by the enzyme is equivalent to its FMN content—that is, 2 moles per mole (*6*).

Dialysis of the orange-red sedimented fractions against water does not restore the original absorption spectrum of old yellow enzyme. However, reduction with $Na_2S_2O_4$ decolorizes the solution, which after anaerobic dialysis against phosphate buffer and reoxidation in air regenerates the old yellow enzyme. This indicates that the proposed complex is very stable

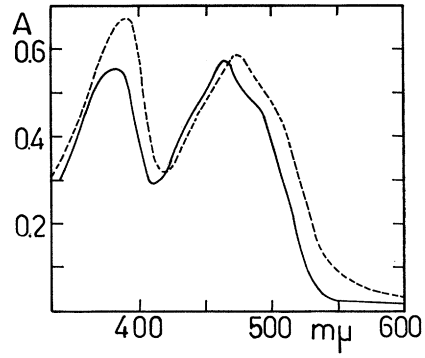

Fig. 1. Absorption spectra of old yellow enzyme (solid curve) and the red complex formed upon reaction with TPNH (broken curve).