Zeitschrift für Klinische Chemie
und Klinische Biochemie

6. Jahrgang September 1968 Heft 5 (S. 357—472)

Functional Disorders of the Brain Induced by Synthesis of Nucleotides Containing 3-Acetylpyridine[1])

By H. Herken[2])

Pharmakologisches Institut der Freien Universität Berlin

(Eingegangen am 3. Mai 1968)

Ernst Schütte zum 60. Geburtstag gewidmet

Experiments on the mechanism of pharmacological effects on the central nervous system have led to the discovery of a lack of specificity of certain enzymes in living cells, which may cause biosyntheses of toxic compounds. R. A. Peters, who observed this phenomenon during his experiments on the toxic effects of fluoroacetate, called it "lethal synthesis". Similar observations can also be made, if antimetabolites of nicotinamide are used, because the lack of specificity of a nucleosidase (NADP-glycohydrolase EC 3.2.2.6) within the endoplasmatic reticulum of the brain cells leads to the biosynthesis of pyridine nucleotides which slow down or completely inhibit the hydrogen transfer between enzyme and substrate of different oxidoreductases. If 3-acetylpyridine is administered to rats in a dose of 80 mg/kg, the formation of nucleotides containing 3-acetylpyridine is the cause of different neurological deficiency symptoms, which include convulsive fits occurring at intervals and permanent disturbances of nervous functions, such as hyperkinesis and ataxic gait. The long lasting deficiency symptoms, which can frequently be shown for more than one year, can be considered as pathobiosis in the sense of the definition given by Heubner.

In isolated brain microsomes, containing a high nucleosidase activity, the synthesis of the dinucleotide phosphate (3-acetylpyridine adenine dinucleotide phosphate) is greater than that of the dinucleotide in the same time and under the same conditions. Spectrofluorometric examinations of brains of rats which had received 3-acetylpyridine also confirmed this preferred biosynthesis of 3-APADP in vivo. The compound was identified in all the brain sections examined, but the highest values were found in the hippocampus of the rat. During the examination of NADP-dependent enzymes the discovery was made, that probably the pentose phosphate cycle is especially concerned. Thereby lower reduction equivalents than in normal cells are made available for the maintenance of the metabolism during the same time unit. The activity of NADP-dependent enzymes of the pentose phosphate cycle is not the same in all brain regions. During the examination of the glucose 6-phosphate dehydrogenase (EC 1.1.1.49) the highest turnover rates were found in the hippocampus.

The kinetics of the malic enzyme (EC 1.1.1.40) discovered by Ochoa are modified in a very peculiar manner, the reaction from malate to pyruvate with 3-APADP acting as hydrogen acceptor being much faster than with the natural coenzyme. The reverse reaction — pyruvate to malate — was greatly slowed down in presence of 3-APADPH_2. 3-APADP can also act as inhibitor in presence of NADP, if more than 50% of the natural coenzyme is present as the derivative of 3-acetylpyridine.

For the comprehension of the long lasting effects of 3-acetylpyridine on the central nervous system, the diminution of the function of the folic acid reductase (EC 1.5.1.3) is probably of importance. Examinations of the RNA-polymerase (EC 2.7.7.6) of the brain nuclei and mitochondria showed that, in principle, an interference with the function of this system is possible. There are certain indications that the biochemical basis of pathobiotic effects induced by drugs is found in disturbances of the function of the cell nucleic acid systems and of protein synthesis. The analysis of the products of metabolism of 3-acetylpyridine has shown that, except for the product of reduction 3-methylcarbinol, no other metabolite can be made responsible for the causation of the central nervous disturbances.

Untersuchungen über den Mechanismus pharmakologischer Wirkungen im Zentralnervensystem haben zu der Entdeckung mangelnder Spezifitäten bestimmter Enzyme in lebenden Zellen geführt, die Synthesen zellschädigender Verbindungen verursachen können. R. A. Peters, der solche Befunde bei seinen Arbeiten über die toxische Wirkung von Fluoracetat erheben konnte, nannte diesen Vorgang eine „tödliche Synthese". Ähnliche Beobachtungen können auch bei der Verwendung von Antimetaboliten des Nicotinsäureamids gemacht werden, weil die mangelnde Spezifität einer Nucleosidase (NADP-Glykohydrolase EC 3.2.2.6) im endoplasmatischen Reticulum der Gehirnzellen zur Biosynthese von Pyridinnucleotiden führt, die den Wasserstofftransport zwischen Enzym und Substrat verschiedener Oxydoreduktasen verlangsamen oder vollständig hemmen. Wenn 3-Acetylpyridin in einer Dosis von 80 mg/kg an Ratten verabreicht wird, so ist die Bildung von 3-Acetylpyridin enthaltenden Nucleotiden die Ursache verschiedener neurologischer Ausfallserscheinungen, zu denen in Intervallen auftretende Krampfanfälle und kontinuierlich bestehende Störungen nervöser Funktionen wie Hyperkinesen und ataktischer Gang gehören. Die lang anhaltenden, oft mehr als ein Jahr nachzuweisenden Ausfallserscheinungen können als Pathobiose im Sinne der von Heubner gegebenen Definition angesehen werden.

Isolierte Mikrosomen des Gehirns, die eine hohe Nucleosidaseaktivität enthalten, synthetisieren mehr 3-Acetylpyridin-adenin-dinucleotidphosphat in der gleichen Zeit und unter gleichen Bedingungen als 3-Acetylpyridin enthaltende Analoge des NAD. Spektrofluorometrische Untersuchungen des Gehirns von Ratten, die 3-Acetylpyridin erhalten haben, bestätigten diese bevorzugte Biosynthese von 3-APADP auch in vivo. Die Verbindung wurde in allen untersuchten Gehirnabschnitten nachgewiesen, doch fanden sich die höchsten Werte im Hippocampus der Ratte. Bei der Untersuchung NADP-abhängiger Enzyme wurde festgestellt, daß der Pentose-Phosphat-Cyclus wahrscheinlich besonders betroffen ist. Dadurch werden in der gleichen Zeiteinheit weniger Reduktionsäquivalente als in normalen Zellen für die Aufrechterhaltung des Stoffwechsels zur Verfügung gestellt. Die Aktivität NADP-abhängiger Enzyme des Pentose-Phosphat-Cyclus ist nicht in allen Hirnregionen gleich. Bei der Untersuchung der Glucose-6-phosphatdehydrogenase (EC 1.1.1.49) wurden die höchsten Umsätze im Hippocampus gefunden.

[1]) These experiments have been supported by a grant by the Deutsche Forschungsgemeinschaft.

[2]) Nach Gastvorlesungen im Department of Biochemistry, University of Oxford/Großbritannien am 5. Juni 1967 und im Department of Neurobiochemistry, Columbia University, New York/USA am 12. Oktober 1967.

Die Kinetik des von OCHOA entdeckten „malic enzyme" (EC 1.1.1.40) wird in sehr eigenartiger Weise verändert, wobei der Umsatz von Malat zu Pyruvat mit 3-APADP als Wasserstoffakzeptor schneller verläuft als mit dem natürlichen Coenzym. Die Umkehr der Reaktion — Pyruvat zu Malat — war in Gegenwart von 3-APADPH$_2$ extrem verlangsamt. 3-APADP kann auch in Gegenwart von NADP als Hemmstoff wirken, wenn mehr als 50% des natürlichen Coenzyms als Derivat des 3-Acetylpyridins vorliegen.
Für das Verständnis der lang anhaltenden Wirkungen von 3-Acetylpyridin auf das Zentralnervensystem ist die Herabsetzung der Funktion der Folsäurereduktase (EC 1.5.1.3) wahrscheinlich von Bedeutung. Untersuchungen der RNA-Polymerase (EC 2.7.7.6) der Gehirnkerne und Mitochondrien ergaben, daß ein Eingriff in die Funktion dieses Systems grundsätzlich möglich ist. Es bestehen gewisse Hinweise dafür, daß die biochemische Basis einer durch Pharmaka erzeugten pathobiotischen Wirkung auf Funktionsstörungen des Nucleinsäuresystems der Zellen bzw. der Proteinsynthese beruht. Die Analyse der Stoffwechselprodukte des 3-Acetylpyridins hat ergeben, daß mit Ausnahme des Reduktionsproduktes 3-Methylcarbinol kein anderer Metabolit für die Erzeugung der zentralnervösen Störungen in Frage kommt.

The analysis of central nervous functions by the use of pharmacological methods has led to the significant result, that there are marked differences in each of the regions of the central nervous system, as far as their sensitivity to chemical substances is concerned. Depending on the chemical structure and efficacy of the chemical compounds, gradually increasing doses not only produce therapeutically useful reactions but frequently also reversible or irreversible damage within the same cells. For this reason, the selective vulnerability of certain cerebral regions is of special theoretical and practical significance, because the examination of chemically induced neurological disturbances can furnish important information for the recognition of the pathogenesis of neurological diseases. Until now, however, the mechanism of such disturbances of cerebral function could only in a few cases be completely elucidated at a molecular level. Successful research into these problems is rendered more difficult by the great variations within the morphological structure of the brain. The best example is still given by the elucidation of the irreversible inactivation of acetylcholinesterase by organophosphorus compounds.

There is no doubt that —as a consequence of its limited permeability — the blood-brain-barrier can inhibit the uptake of noxious substances into certain cerebral regions. The methods of autoradiography, however, have more and more proved the fact, that several chemical compounds can be accumulated to a considerable extent within the individual cells of the brain. The accumulation of a chemical substance within a certain cerebral region does not implicitly mean that this substance will produce its greatest activity there. The accumulation of a drug gains special interest, whenever it affects the metabolism of the cells concerned, gives rise to enzymic dysfunctions, and these in turn are the real cause of more serious damage.

Studies of the chemical basis of pharmacological actions of drugs have also led to the discovery of a peculiar lack of specificity of certain enzymes, thus enabling living cells to synthesize harmful substances. This is not in accordance with the general opinion on the strongly specific function of enzymes. The first observation of the lack of specificity of important enzymes in intermediary metabolism was contributed by R. A. PETERS (1) in the course of his studies on the toxic action of fluoroacetate. The term "lethal synthesis" used by R. A. PETERS (1) in his Croonian Lecture before the Royal Society to define this process is very well justified, because the animals died of the metabolic disturbances within the mitochondria of the brain, developing generalized convulsions and apnea.

In this case the chemical basis of drug action is relatively simple and clear. Far more difficult to analyze are, of course, those effects, the chain of reactions of which is longer, thereby preventing a clear-cut survey. This especially holds for effects of the more chronic type which are characterized by irreversibility or a slow restoration of the functional impairment. During the experiments we carried out on the effects of antimetabolites of nicotinamide, we observed special kinds of central nervous disturbances which, in the German scientific literature, are generally being referred to as pathobiosis.

The term was introduced by WOLFGANG HEUBNER (2) in 1922, while addressing the Göttingen Scientific Society. He used the term "pathobiosis" to define a characteristic form of long lasting toxic effects of drugs in living tissues. In general, he used this term for all disturbances of cell functions ranging from fully reversible to irreversible ones which do not entail an entire necrosis of cells. He also included the different degrees of deviations from normal, the duration of which need not be linked with the presence of a drug. The chronic effects after acute intoxication, the long lasting effects of X-rays, the production of malignant tumors by carcinogenic substances and the teratogenic properties of certain drugs may be classified as pathobiosis.

Although many experiments have been made to explain the chemical basis of pathobiotic effects induced by drugs especially in the central nervous system, our knowledge is as yet very poor. During the past few years we have carried out studies on antimetabolites of nicotinamide and have been able to look into the biochemical mechanism of such an effect. These observations also gave information for better understanding of the selective vulnerability of certain regions of the brain.

By means of this example I shall later try to define the mechanism of "pathobiosis" as it may be understood today from the pharmacological point of view. The antimetabolite of nicotinamide by means of which I shall first explain this principle, is 3-acetylpyridine. Its structure very much resembles that of nicotinamide (Fig. 1).

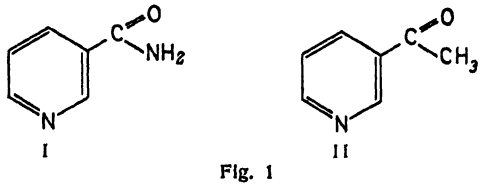

Fig. 1
Chemical Structures of Nicotinamide (I) and 3-Acetylpyridine (II)

The drug was injected into the rat at a dose level of 80 mg/kg. After a latent period of 2 to 3 hours, intoxication gradually increased and during this time the effect on the central nervous system became markedly evident. After a period of paralysis a condition of excitement gradually developed which was made evident by constant rolling cramps and by massive loss of equilibrium. At this stage some of the animals died. Among the survivors, chronic effects were evident which may be seen as hyperkinesis, disturbance of muscular coordination with ataxia and at intervals tonic cramps.

The disorientation of motions revealed, that each movement of a limb is carried out twice in a nearly regular rhythm, only after a reaction which resembles an uncertain wiping motion. The rat places the paw firmly on the surface and this produces an insecure staggering movement. Occasionally the animals move with stiff legs. In addition, between the attacks of cramps which appear spontaneously without visible cause, the animals show abnormal behaviour. The rats tremble in panic and try to evade by scrambling backwards. Hereby a heavy tremor of the forelegs with fibrillation of the muscles may occur. The animals survived these effects on the central nervous system caused by a single injection of 3-acetylpyridine for several months or sometimes for more than a year.

If more common criteria are to be used to explain the disturbances, we can distinguish between *symptoms* which appear as *attack at intervals* and those that occur *continuously* with losses of general nervous function.

Electroencephalographic studies reveal definite pathological changes. 4 hours after application of 3-acetylpyridine a general increase in the amplitude of the EEG waves is found, which show rhythmical and generalized seizure charges.

To begin with, I should like to talk on the biochemical basis of this intoxication. KAPLAN and his team (3, 4) during their investigations proved that 3-acetylpyridine can be exchanged for nicotinamide in NAD and NADP by the action of a nucleosidase (NADP-glycohydrolase, EC 3.2.2.6). This enzyme, besides being capable of hydrolysing, also has transferase activity, which, however, is not specific. Thus by an erroneous action compounds resembling nicotinamide, such as 3-acetylpyridine, may be transferred to adenosine diphosphate ribose.

The nucleosidase is almost exclusively present in the endoplasmatic reticulum of the cell. It cleaves only the oxidized pyridine nucleotides between the ribose and the nitrogen of the pyridine derivative. The reduced compounds are not split, as first shown by MCILWAIN and RODNIGHT (5a, b).

It is possible that the positively charged nitrogen of the oxidized pyridine compound reacts with an active centre on the nucleosidase. Figure 2 shows the scheme of hydrolysis and the exchange of nicotinamide for acetylpyridine. Following the linkage of NAD(P) to the enzyme, a hydrolysis in nicotinamide and adenosine diphosphate ribose will take place. Nicotinamide is an inhibitor of this reaction, so that the hydrolysis is slowed down with growing concentration of free nicotinamide. The exchange reaction will remain untouched, and adenosine diphosphate ribose can serve as acceptor for 3-acetylpyridine and nicotinamide. The concentration of 3-acetylpyridine should at least be 10 times higher than that of nicotinamide for the synthesis to occur. The competitive antagonism will explain, why the onset of the toxic symptoms can also

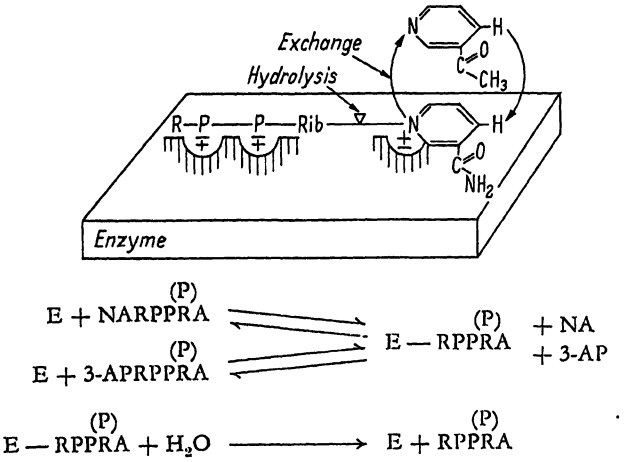

Fig. 2
Hydrolysis of NAD(P) and Synthesis of 3-APAD(P) by Nucleosidase (Glycohydrolase) from the Endoplasmatic Reticulum

be prevented in vivo by the early injection of nicotinamide. The scheme shows, that the lack of specificity of the nucleosidase also allows the synthesis of abnormally structured nucleotides. The synthesis presumes, that unchanged NAD or NADP are available. The final hydrolytic cleavage of adenosine diphosphate ribose from the enzyme at the same time ends the transfer reaction.

A 3-acetylpyridine derivative of NAD was first isolated from tumors by KAPLAN, GOLDIN, HUMPHREYS, CIOTTI and VENDITTI (6). Assays carried out by the same investigators to demonstrate the same substance in the brain were unsuccessful. This failure might have been due to the fact, that the method employed by KAPLAN was not suitable. In 1962 we were able to isolate a nucleotide containing 3-acetylpyridine from the brain of intoxicated animals (COPER and HERKEN) (7). This substance was purified by chromatography and identified by spectrophotometric and enzymic procedures. But, the amount was so small that it was impossible to ascribe the loss of the neurological functions to the activity of this substance. Only 4 to 6% of the natural NAD was converted into the compound containing 3-acetylpyridine. In addition the substance

acted as hydrogen acceptor and donor with different dehydrogenases so that a severe inhibition of the metabolism by this nucleotide is unlikely.

In isolated brain microsomes, containing high nucleosidase activity, the synthesis of the dinucleotide phosphate (3-acetylpyridine adenine dinucleotide phosphate) is greater than that of the dinucleotide in the same period and under similar conditions (Brunnemann, Coper and Herken) (8) (Fig. 3).

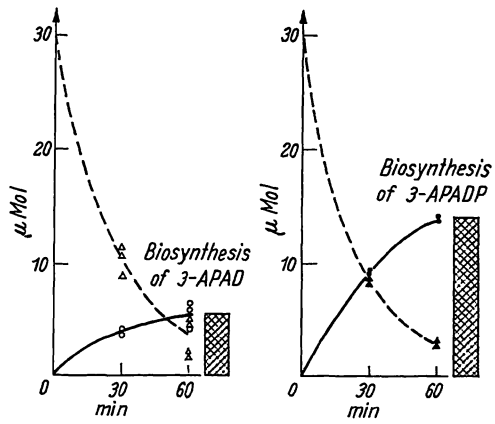

Fig. 3
Hydrolysis of NAD(P) and Synthesis of 3-APAD(P) by Nucleosidase (Glycohydrolase) from Brain Microsomes (0.1 M 3-AP)

△ — — — △ Hydrolysis of NAD
▲ — — — ▲ Hydrolysis of NADP
o ——— o Synthesis of 3-APAD
• ——— • Synthesis of 3-APADP

The presence of 3-APADP in the brain cells of the intoxicated animals was difficult to prove for two reasons:

1) The concentration of NADP in the brain is 15 to 20 times lower than that of NAD.

2) The sensitivity of the methods used was not sufficient for the determination of the quantity of the nucleotides containing 3-acetylpyridine present in the special regions of the brain in the presence of the naturally occurring coenzyme.

Lowry, Roberts and Kapphahn (9) developed a highly sensitive spectrofluorometric method for the determination of NAD (NADP) in concentrations of 10^{-11} mol/ml. It is based upon the treatment of the nucleotides with 6N NaOH. It cannot be applied to the identification of nucleotides containing 3-acetylpyridine, as these nucleotides do not fluoresce under these conditions. Burch, Storwick, Bicknell, Kung, Alejo, Everhardt, Lowry, King and Bessey (10) found that still more intensive fluorescence reactions can be produced by addition of methyl ethyl ketone. Applying this method for the fluorometric determination of 3-APAD, 3-APADP, and 3-acetylpyridine, we found that with acetone as well as with methyl ethyl ketone fluorescent compounds can be produced which clearly differ from the nucleotides NAD, NADP and 1-methylnicotinamide.

Amongst the ketones tested (acetone, methyl ethyl ketone, phenyl methyl ketone, acetylacetone) methyl ethyl ketone has proved to be the most efficient. During

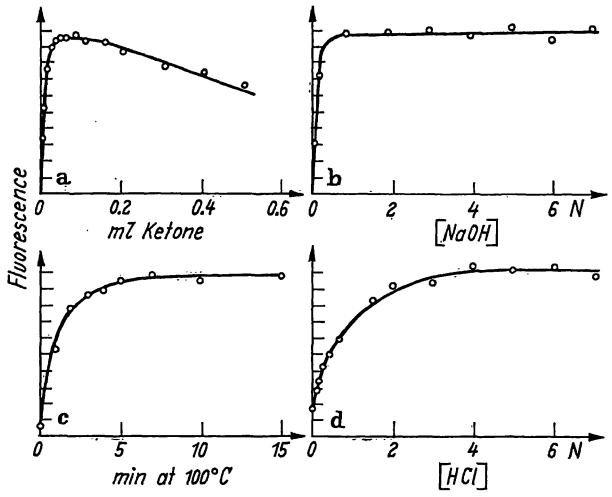

Fig. 4
Dependence of the APAD-(APADP)-Fluorescence on the Quantity of Methyl Ethyl Ketone (a), of the Base Concentration (b), of the Incubation Time in Alkaline Medium (c), and of the Acid Concentration (d). 3.3 µg APAD have been employed. (Herken and Neuhoff) (11)

these experiments the following observations were made (Fig. 4): methyl ethyl ketone only yields a fluorescent product with 3-APAD (3-APADP) in presence of NaOH. The optimal reaction is obtained with 0.1 ml 1N base and remains unchanged up to concentrations of 7N NaOH (Fig. 4b). Immediate heating of the thoroughly shaken reaction mixture for 7 min at 100° C will produce a more intensive fluorescence than longer incubation at lower temperatures. A maximum fluorescence will be obtained by subsequent addition of 1 to 2 ml 5N HCl and by renewed incubation for 5 min at 100° C. An increase of the acid concentration has no effect (Fig. 4d). The final product of the reaction is cooled to room temperature, diluted, and measured in the Aminco-Bowman-spectrofluorometer. The product of the reaction must be kept in the dark until it is measured, as longer exposure to light will cause a decrease of the fluorescence. The method is highly sensitive and permits an accurate differentiation of the natural coenzymes NAD and NADP from the derivatives containing 3-acetylpyridine. Even the presence of other derivatives of nicotinamide did not interfere with this method (Herken and Neuhoff) (11).

The excitation spectra of NAD and 3-APAD (3-APADP) show the differences between the natural and the abnormally structured products. For those nucleotides which contain 3-acetylpyridine, there is a second characteristic maximum occurring at a wavelength of 265 nm. The maximum emission is at 500 nm. This emission is used for the determination of the concentration of the compound in the brain. The high sensitivity of this method made it possible to demonstrate the presence of nucleotides containing 3-acetylpyridine up to a range of $2 \cdot 10^{-12}$ mol present in 1 mg of wet weight of brain cells. This also applies to NADP and 3-APADP, even when only 1 to 2% of the natural coenzyme was converted into the compound containing 3-acetylpyridine (Fig. 5) (Herken and Neuhoff) (11).

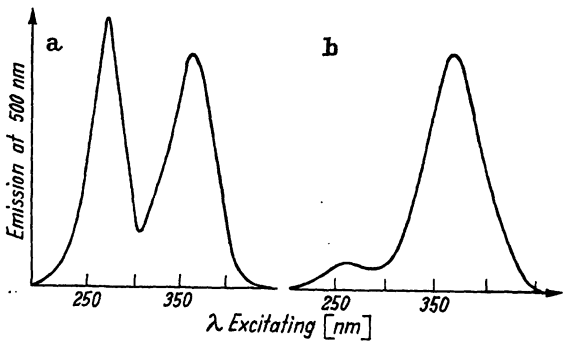

Fig. 5
Specific Excitating Spectra of the Reaction Products of 3-APAD(P) (a) and NAD(P) (b) with Methyl Ethyl Ketone
a) $5 \cdot 10^{-8}$ Moles 3-APAD, 3-fold amplified
b) $5 \cdot 10^{-8}$ Moles NAD, 35-fold amplified

The derivative of NAD containing 3-acetylpyridine which is of minor interest, and the natural coenzyme were destroyed after enzymic hydrogenation with alcohol dehydrogenase and subsequent treatment with perchloric acid. During this reaction, the oxidized part of NADP and also of 3-APADP was retained in the extract which made possible a quantitative determination of these nucleotides by the spectrofluorometric method.

Extraction of the whole brain furnished the surprising evidence that nearly 40% of naturally occurring NADP is converted into an abnormally formed compound containing 3-acetylpyridine. NEUHOFF (12) has found in our Institute, that the concentration of 3-acetylpyridine in the brain 4 hours after injection is 5 to 10 times higher than that of nicotinamide. The establishment of a concentration gradient against the extracellular space indicates an active transport of 3-acetylpyridine through the cell membrane, and the formation of abnormally structured nucleotides reaches the maximal value at about the same time. Using spectrofluorometric methods, it is possible to determine the biosynthesis of 3-APADP within 9 different regions of the brain. In all of them, the abnormally structured nucleotide was, however, proved to be present in different concentrations.

We found that the exchange of 3-acetylpyridine with nicotinamide reaches the maximum value in the NADP of the hippocampus (Fig. 6). This finding is interesting for the reason that it possibly explains the observed neurological disturbances, which occur in certain intervals as convulsive fits. As is well known, the hippocampus plays a dominating role for the regulation of excitation processes in the brain, and this as part of the limbic system. This probably does not cause the continuous disturbances of the movements previously described. They have more to do with the extrapyramidal system.

It is striking, that the amount of NADP as compared with the controls was not diminished as would be expected from the formation of 3-APADP, by the exchange of nicotinamide for 3-acetylpyridine. These deviations can either be the expression of a change of a redox quotient in favour of the oxidized form of the nucleotides or can be due to an additional formation of the abnormal nucleotide. This has not been investigated.

During the examination of the cell fractions, we observed that almost nothing is formed in the mitochondria, but more than 90% was found in the cytoplasm of the brain cells which did not include the mitochondria. Since, according to KLINGENBERG (13) nearly 35% of the total NADP of the brain cells is present in the mitochondria, and this is not converted, the proportion of 3-acetylpyridine adenine dinucleotide phosphate in the remaining part of the cell increases correspondingly.

The preference for the hippocampus can also be confirmed by autoradiography (HERKEN) (14, 15). After injection of ^{14}C-labelled 3-acetylpyridine, the strongest radiation was found in certain definite regions of the hippocampus. The method used does, however, not permit more detailed differentiation within the cells.

On the other hand, a comparison with a picture of the topographic anatomy of the hippocampus makes evident, that the "stratum pyramidale" and perhaps the marginal areas of the "stratum oriens" and the "stratum radiatum" in the regions CA 2 and CA 3 are

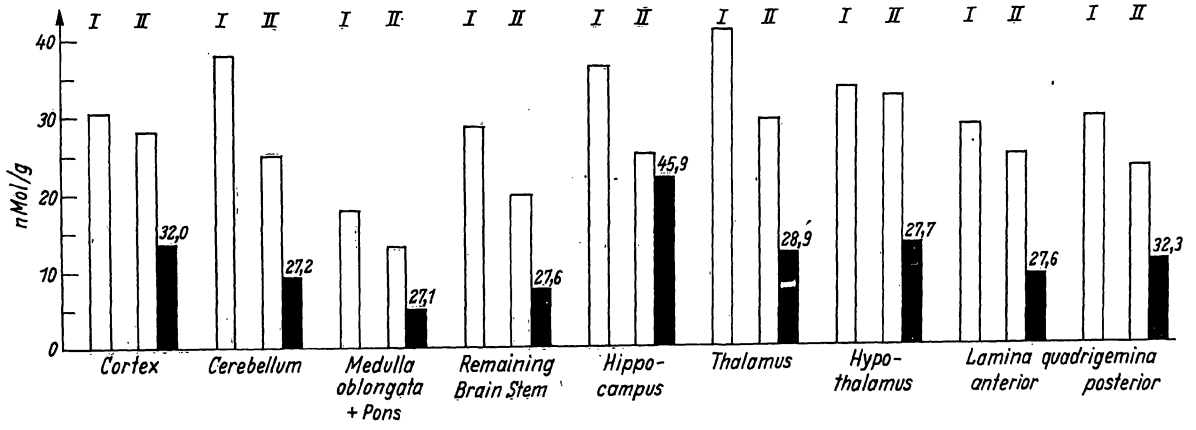

Fig. 6
NADP and 3-APADP Content in Different Brain Regions I. Control II. 6—7 hrs after 100 mg/kg 3-AP, Numbers = 3-APADP in % of 3-APADP + NADP
□ = NADP ▨ = 3-APADP

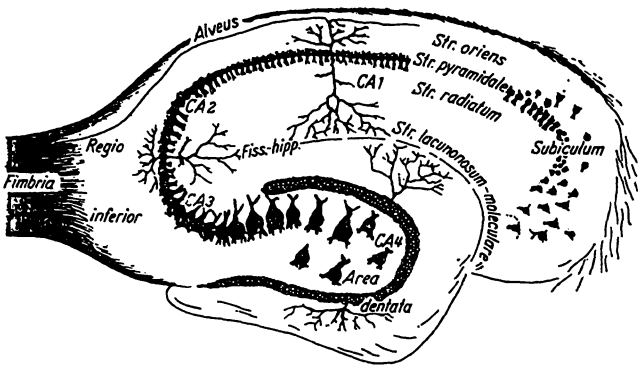

Fig. 7
Topographic Drawing of Hippocampus, schematically according to data published by BLACKSTEAD and ANDERSEN (37, 38)

obviously involved. These are areas which are commonly assumed to be metabolically highly active (Fig. 7).

The electron micrograph of the nuclear region of a pyramidal cell demonstrates clearly, how closely the nucleus is surrounded by the endoplasmatic reticulum, in which the NAD- and NADP-nucleosidases are localized. This can explain the high degree of transfer reactions in this area. I take this opportunity to thank Dr. NIKLOWITZ of the Institute for Brain Research of the Max-Planck-Society, Frankfurt, for his electron micrograph. The close spatial relationship between the ergastoplasm and the nucleus probably also permits the exchange of nucleotides between these cell structures through the pores of the nucleus, possibly resulting in a disturbance of the metabolism of the nucleus (Fig. 8) (NIKLOWITZ and BAK) (16).

What are the consequences of the accumulation of 3-acetylpyridine dinucleotide phosphate to the metabolism of the brain cell? O. WARBURG (17) has found: "Die Wirkung der Pyridinnucleotide in der lebendigen Substanz beruht auf der Fähigkeit ihres Pyridinanteiles, zwei Wasserstoffatome zu addieren und diesen Wasserstoff wieder abzugeben, also auf der Fähigkeit, reversible Dihydropyridinverbindungen zu bilden". The replacement of nicotinamide by 3-acetylpyridine raises the question, whether there occurs a disturbance of hydrogen transfer between enzymes and substrates using pyridine nucleotides as co-factors. Almost nothing was known about the behaviour of 3-APADP in such systems. There only exists an indication from the work of KAPLAN, CIOTTI, and STOLZENBACH (4), that the isocitrate dehydrogenase (EC 1.1.1.42) obtained from the heart of pigs reacts significantly slower with this unnatural pyridine nucleotide than with the natural coenzyme.

Hence, we prepared 3-APADP with the help of microsomal enzymes and studied the kinetics of the hydrogen transfer in different dehydrogenases in vitro (COPER and NEUBERT; NEUBERT and COPER; HERKEN and TIMMLER) (18, 19, 20, 21). The study revealed, that all NADP-dependent enzymes have a retarded ability for hydrogen transfer, if they are incubated with 3-acetylpyridine adenine dinucleotide phosphate instead of with the natural coenzyme. While studying the kinetics of such enzymes COPER and NEUBERT found an exception, to which I shall refer later on. Yet, there were significant differences in the extent to which these reactions were affected; they are illustrated in Figure 9.

Fig. 8
Section of a Pyramidal Cell (Region CA 2) with Nuclear Cap cut longitudinal
ER(K) = rough endoplasmatic reticulum at head of the nucleus
NM = nuclear membrane
NP = nuclear membrane pore
GoK = Golgi complexes
M = mitochondria
GoB = Golgi vacuole
GrV = granula
magnification: × 26000 (NIKLOWITZ and BAK) (16)

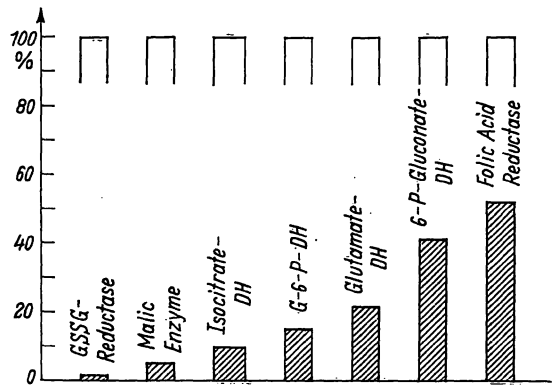

Fig. 9
Velocity of Reactions of different Dehydrogenases with 3-APADP(H_2) as Co-Factor, calculated from Lineweaver-Burk-Diagrams
NADP(H_2) = 100%

It was of particular interest to note, that the first two NADP-dependent steps of the pentose phosphate cycle are involved, and these are important for the production of the reduced coenzyme. The enzymes concerned are glucose 6-phosphate dehydrogenase (EC 1.1.1.49) and 6-phosphogluconic acid dehydrogenase (EC 1.1.1.43). If the first enzymic step of the Warburg-Dickens-Horecker cycle is reduced to 1/6 of the original turnover after conversion of NADP into the nucleotide containing 3-acetylpyridine, it will also have obvious unfavourable consequences on the following reaction. At the same time, the reduction-equivalents, which are necessary for the maintenance of normal cell function, are greatly lowered.

The activity of NADP-dependent enzymes taking part in the pentose phosphate cycle is not the same in

different brain regions. BRUNNEMANN and COPER (22) of our Institute have found, that the hippocampus holds a special position in having the highest turnover of glucose 6-phosphate dehydrogenase.

The investigation of different enzymes with 3-acetylpyridine adenine dinucleotide phosphate as coenzyme showed that each reaction which leads to a change in the redox quotient in favour of the oxidized form of pyridine nucleotides, will also influence the hydrogen acceptor system. There is, e. g., a very strong inhibition of glutathione reductase at different concentrations of the substrates.

Based on these results, the following scheme can be drawn up (Fig. 10). At the left, the hydrogen donors are shown and at the right the hydrogen acceptors. The diminution of the reduction equivalents by a slow

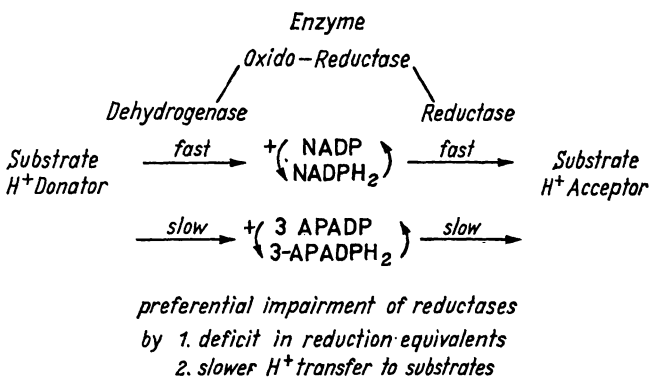

Fig. 10
Schematic Representation of H+-Transfer between Substrate and Enzyme with NADP and 3-APADP as Co-Factors

hydrogen transfer in presence of 3-APADP as coenzyme of the dehydrogenase indirectly leads to a reduction of the efficiency of reductases. This, however, presupposes, that the mentioned enzymes are directly connected with each other in one compartment of the cell.

During their investigations concerning the velocity of the hydrogen transfer between substrate and NADP-dependent enzymes, NEUBERT and COPER (20), while studying the malic enzyme (EC 1.1.1.40) which had been discovered by OCHOA, obtained some results which may contribute towards the understanding of the acute neurotoxicity of 3-acetylpyridine.

The measurement of the kinetics showed that the reaction from malate to pyruvate, with 3-APADP acting as hydrogen acceptor, was faster than that with the natural coenzyme NADP (Fig. 11). As yet, we have not found this with any other NADP-dependent enzyme.

The reversal of the reaction, pyruvate to malate, in presence of 3-APADPH$_2$ was extremely slow. This is shown on Figure 12.

3-APADP can act as inhibitor in presence of NADP which entails that the catalysis can also be slowed down in vivo, if more than 50% of the natural coenzyme is present as derivative of 3-acetylpyridine. This has e. g. been proved in the hippocampus (Fig. 13).

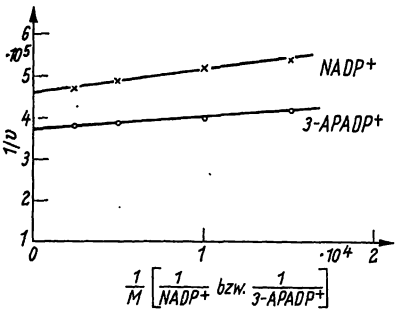

Fig. 11
"Malic Enzyme"-Reaction (Malate ⟶ Pyruvate) from Rat Brain. Comparison of reaction rates in the presence of NADP+ or 3-APADP+

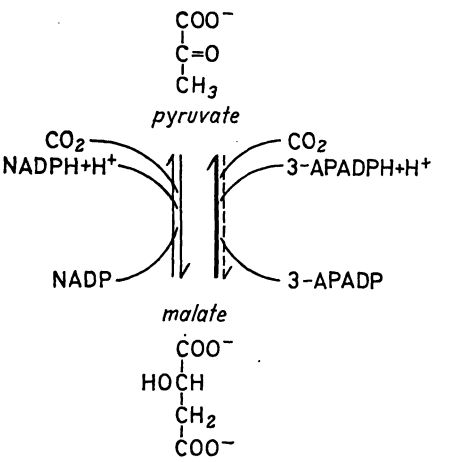

Fig. 12
"Malic Enzyme"-Reaction with NADP(H$_2$) or 3-APADP(H$_2$) as Coenzymes

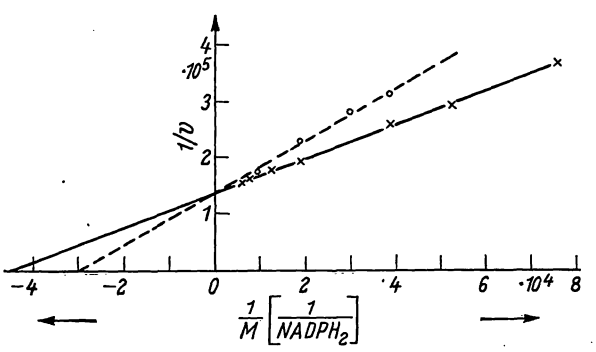

Fig. 13
Inhibition of the Activity of the "Malic Enzyme" by 3-APADPH$_2$
o— — —o NADPH$_2$ + 50 μM 3-APADPH$_2$ ×——× NADPH$_2$

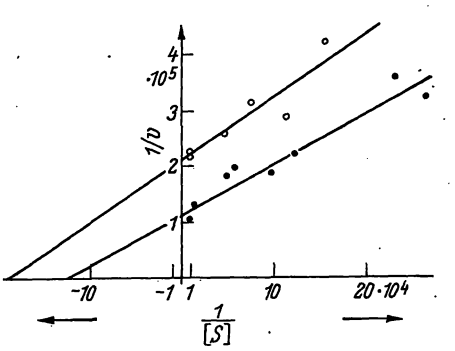

Fig. 14
Comparison of FH$_2$ Reductase Activities from Rat Brain with NADPH + H+ (•—•) and 3-APADPH + H+ (o—o) as Hydrogen Donator. Transformation of dihydrofolic acid into tetrahydrofolic acid registered by the decrease in extinction at 340 nm
Measuring cuvette contained: 1 mM $MgCl_2$, 1 mM isocitrate, 0.2 ml isocitrate dehydrogenase, a suitable amount of the enzyme preparation, M/15 phosphate buffer pH 7.5, NADP or 3-APADP in various concentrations listed on the diagram

For the understanding of the long lasting effects of 3-acetylpyridine, the conversion of dihydrofolic acid into tetrahydrofolic acid which is slowed down in presence of 3-APADP is probably important.

The evaluation of Lineweaver-Burk-diagrams showed a significant dependence of the reaction velocity of the enzymic reduction of the folic acid on the amount of NADPH present (Fig. 14). This reduction was specifically dependent on this pyridine nucleotide; the incubation with NADH was ineffective. The replacement of the natural coenzyme by the derivative containing 3-acetylpyridine slows down the velocity of the conversion of FH_2 to FH_4 to approximately one half of the natural value. For judging the process in vivo, it is, of course, important to resolve the problem, as to whether the quantities are sufficient to prevent the activity of natural coenzymes that are still present. The specific consequences of the above-mentioned reaction may result in the formation of activated formate and in the transfer of C 1 units during the biosynthesis of important products of intermediary metabolism, such as serine, methionine, choline, and also purines and pyrimidines. This specificity depends on particular localization and the degree of synthesis in different cells of the brain. The de novo synthesis can be of special importance for those compounds, which cannot pass the blood-brain-barrier.

After injection of 30 μC of ^{14}C-labelled formaldehyde, distinctly marked regions in the hippocampus have been found by KUSCHINSKY (23). The greatest activity is found within the regions CA 2 and CA 3 as well as in the gyrus dentatus, as can be seen in the hippocampus in a survey autoradiograph of the whole brain. Also in the brain, formaldehyde is evidently utilized in the synthesis of nucleic acids and proteins. Other authors, especially RICHTER and his team (24), have found, that after the application of ^{14}C-labelled glucose, considerable amounts of the labelled carbon could be traced in the proteins of the brain cells. Some experiments, which we carried out in the meantime for the purpose of orientation with uniformly ^{14}C-labelled glucose, indicate that the synthesis of 3-APADP in the ganglion cells exerts a lasting influence on such processes of the metabolism in vivo. 18 hours after intoxication only about 60% of the activity, which could be traced in untreated animals, was found in the proteins of the cell nuclei.

In order to answer the question whether the symptoms of deficiency are caused by the incorporation of 3-acetylpyridine alone, it was important to determine which metabolites of acetylpyridine are produced in the organism. Laboratory examinations of the urine have proved, that no free acetylpyridine is eliminated. BEHER and his team (25) had already obtained this result at an earlier date. These experiments performed in our Institute by NEUHOFF, HARRIS, and KÖHLER (26, 27) showed that in metabolism numerous compounds are derived from 3-acetylpyridine; 12 of them could be isolated and identified. The first metabolites known were

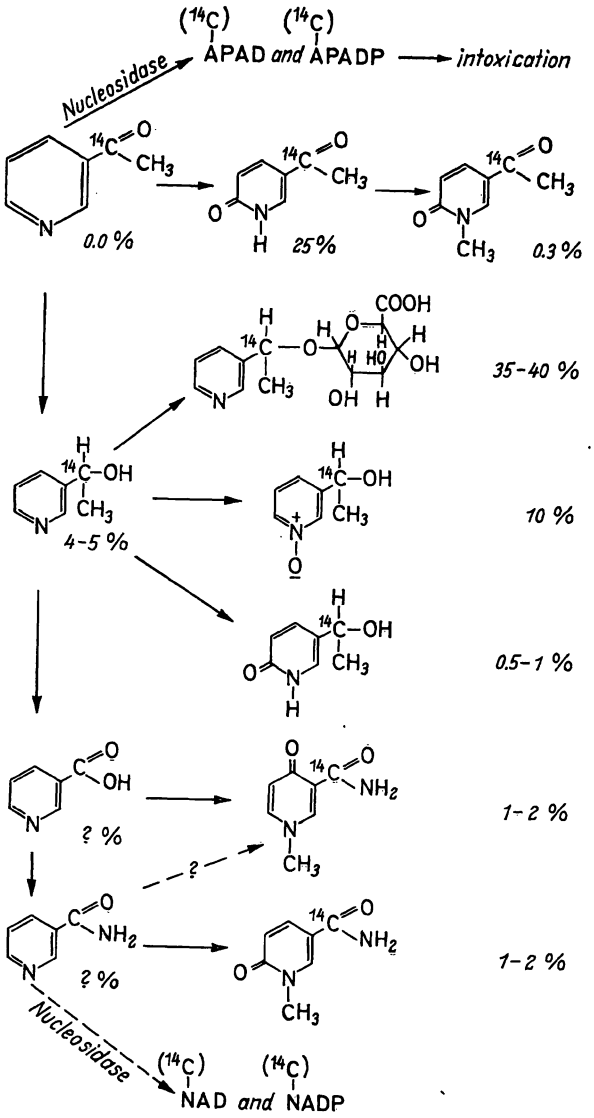

Fig. 15
Metabolism of 3-Acetylpyridine
(% = ^{14}C-excretion in the urine after intraperitoneal application of ^{14}C-3-acetylpyridine)

N-methyl nicotinamide and nicotinic acid. Later on, pyridine 3-methylcarbinol and pyridine 3-methylglycol followed.

Figure 15 shows all those compounds which could be isolated and identified from urine after administration of acetylpyridine. The main product of transformation seems to be 3-methylcarbinol, produced by reduction; according to the results of NEUHOFF and KÖHLER (27), this product is very quickly glucuronized in the organism. 35 to 40% of the activity of ^{14}C-labelled 3-acetylpyridine given by injection was attributed to the glucuronide. The percentage of the remaining metabolic products may be seen from the figure. Only a small percentage of the applied activity is attributed to further metabolic products; these are derivatives of nicotinic acid and nicotinamide, also originating from 3-acetylpyridine.

Of all the pyridine compounds hitherto isolated, only pyridine methylcarbinol possesses a pharmacological activity which approximately corresponds to that of 3-acetylpyridine. As far as can be determined, however,

the consequences do not seem to be so long lasting. This compound, too, is transferred into the pyridine nucleotides of the brain. Among the further compounds which — due to their structure — can be transferred, 3-acetylpyridine 6-pyridone, and 3-methylcarbinol 6-pyridone were entirely non-toxic. According to our present knowledge, the same applies for pyridine 3-methylcarbinol glucuronide.

There exist basic differences between the biosynthesis of other acetylpyridine derivatives in vitro and in vivo. 2-Acetylpyridine is not transferred to NADP by the nucleosidase of the microsomes of the brain, neither in vitro nor in vivo. In vitro, 4-acetylpyridine is exchanged for nicotinamide faster than 3-acetylpyridine, so that considerable quantities of this nucleotide could be isolated. As was to be expected from the structure, this compound was ineffective in dehydrogenase- or reductase-systems. A completely contrary observation was the fact that the toxicity of 4-acetylpyridine is very low. Symptoms of excitement with generalized convulsions are not seen, even at a dosage of 1 g/kg. The probable reason for this phenomenon is the assumption, that 4-acetylpyridine will be changed very quickly in the metabolism of the liver, or cannot penetrate the cells of the hippocampus. We could not find any nucleotide containing 4-acetylpyridine in the brain.

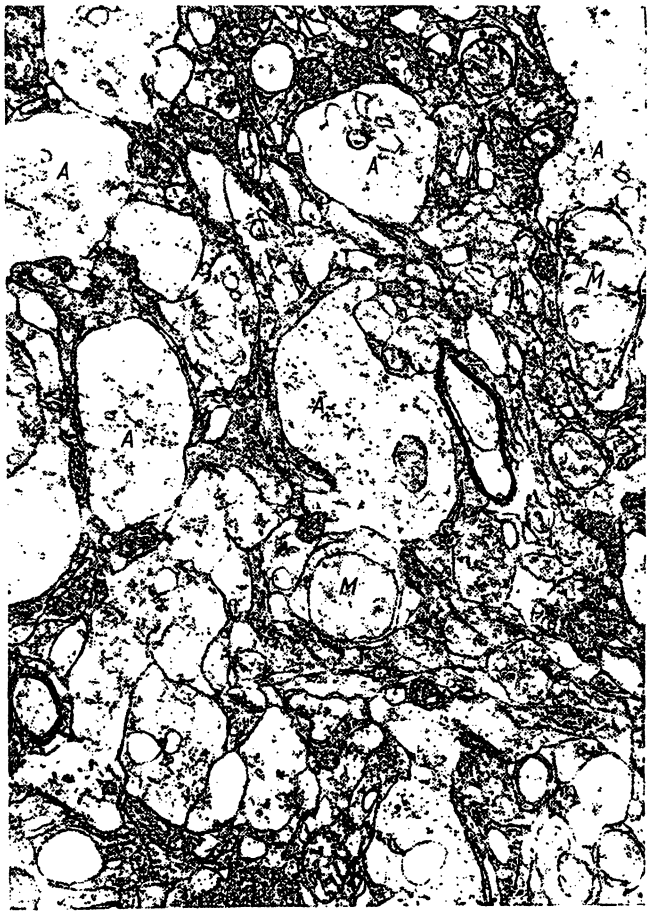

Fig. 16
Archipallium of the Rat after Application of 3-Acetylpyridine
A = astrocytes with hydropic swellings
M = mitochondria
Magnification: × 42000 LIERSE (32)

Fig. 17
Appendix of Astrocyte near the Vessels with Granular Enclosure (1) Containing Residual Membranes (arrow). 2 = mitochondria, 3 = basal membrane. Cerebral cortex of the rat 1 year after intoxication with 3-acetylpyridine. Magnification: × 58200. LIERSE (32)

Considering the intensity and severity of recognizable symptoms of intoxication, we would expect a definite destruction of the morphological structure in certain zones of the brain. HICKS (28), and also COGGLESHALL and MCLEAN (29, 30) reported microscopically recognizable lesions in the hippocampus of rats. The existence of such lesions could neither be confirmed by MCLEAN himself (31) nor by LIERSE (32) and PETERS (33) who used our experimental animals. Obviously there is a big difference in the sensitivity of the brain of different species.

Electron microscopic studies of the ultrastructure of the brain after the administration of 3-acetylpyridine revealed changes mainly in the astrocytes, oligodendrocytes, and microglial cells with an enlargement of the ergastoplasm. It was, however, difficult to interpret the morphological changes in these animals living one year after acute intoxication and still showing symptoms of a defective central nervous system, and convulsions.

In addition to the almost regularly recognizable hydropic swelling of astrocytes (Fig. 16) which might have resulted as a consequence of convulsions, changes in the Golgi apparatus were very often noticed.

Homogeneous or granulated particles in the nucleus and outside were particularly prominent (Fig. 17). According to LIERSE (32), such particles cannot be found in such a frequency among normal animals of similar age.

It is difficult to say, whether these changes are responsible for the long lasting central nervous system symptoms. The answer to this question is made more difficult, because we have to deal with symptoms which, neurologically, are very different. In the meantime, further work on histological changes in the brain of the

rat after 3-acetylpyridine has taken its effect, has been published; this describes severe lesions of the lower part of the oliva. They may be responsible for the continuing disturbances of movement. Also the rolling cramps which are found during the acute stage of the intoxication and the observed disorders of balance point to the fact, that perhaps the medial section of the oliva is also affected.

These findings do not, however, explain the development of the paroxysmal convulsions, which, with several animals were found still several months and even 1 year after one single application of 3-acetylpyridine. DENK, HAIDER, KOVAC and STUDYNKA (34) also noted defects in the ability of learning with the animals intoxicated with 3-acetylpyridine, but were unable to obtain sufficient neuropathological evidence to explain them. It is, therefore, not impossible, that these symptoms are caused by a special kind of disturbance within the molecular area.

As the synthesis of 3-acetylpyridine adenine dinucleotide is completed after 24 hours, and 3-acetylpyridine at this time is quantitatively eliminated, it is evident that during the first 24 hours of intoxication, a metabolic disturbance is produced bringing about a continuous reproduction of a biochemical lesion and forming the cause of pathobiosis. Such an assumption leads us to look for the cause of the disturbance in the area of nucleic acid polymerase or of protein synthesis depending on nucleic acid function.

Experiments on the RNA-polymerase (EC 2.7.7.6) of the brain nuclei and mitochondria, carried out in our

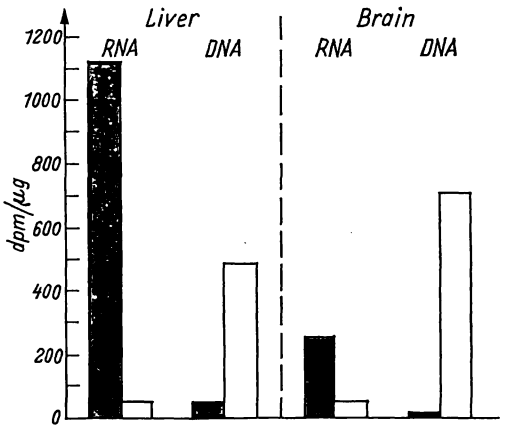

Fig. 18
Activity of RNA-Polymerase in Isolated Nuclei (■) and Mitochondria (□) of Rat Liver and Brain
Incubation time: 5 min.
The incubation tube contained: 80 mM Tris-HCl, 15 mM phosphate buffer pH 8.0; 50 mM KCl; 5 mM $MgCl_2$; 0.5 mM EDTA; 8 mM GSH; 0.4 mM ATP, CTP, GTP; 14.8 µC ^{3}H-UTP. Nuclei isolated following the method of WIDNELL and TATA (36). Mitochondria purified by sucrose gradient floatation

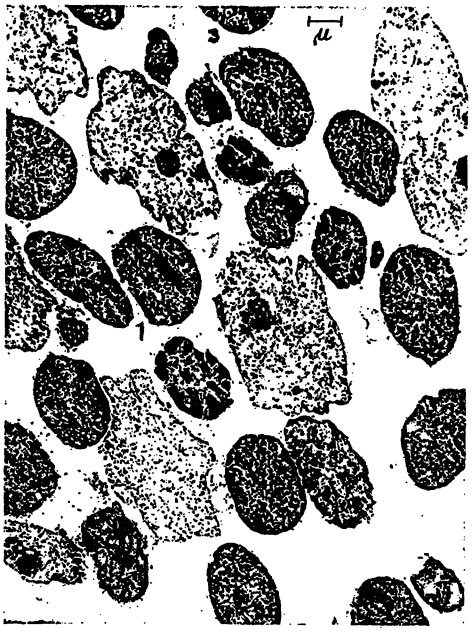

Fig. 19
Cell Nuclei Isolated from Rat Brain According to the Method of WIDNELL and TATA (36). The large cell nuclei can be associated with ganglia cells, the smaller ones with the different types of glia cells. The cell fraction appears electron microscopically pure. Magnification: × 8000. MERKER (1967)

Institute by NEUBERT and OBERDISSE (35) show, that pharmacological changes in two different nucleic acid systems of the cell are possible. The absolute turnover of the RNA is much higher in the cell nuclei (Fig. 18).

An electron micrograph shows that the examined nucleus fraction of the brain is pure (Fig. 19). Morphologically the nuclei are, however, not uniform. Probably the lighter and bigger cell nuclei come from ganglion cells and the smaller ones from the glia cells. We hope to be able to separate these two types from each other by centrifugation in a density gradient, in order to facilitate the localization of the lesion.

If the RNA-polymerase activity in the cell nuclei and in the mitochondria is expressed in terms of the DNA present, the turnover rate is relatively higher in the mitochondria than in the nuclei. This is valid for the liver as well as for the brain. The limitation of the efficiency of the folic acid reductase (EC 1.5.1.3) can disturb the synthesis of thymidine, but satisfactory results on the exchange of thymidine in the DNA of different brain cells are still lacking. There are, however, many indications, that the biochemical basis of pathobiotic action induced by drugs is found in disturbances of the function of the cell nucleic acid systems or in protein synthesis regulated by messenger RNA.

References

1. PETERS, R. A., Proc. Roy. Soc., London, Biol. Sc. *139*, 143 (1952). — 2. HEUBNER, W., Nachr. Akad. Wiss. Göttingen. math.-physik. Kl. (1922). — 3. KAPLAN, N. O. and M. M. CIOTTI, J. Amer. chem. Soc. *76*, 1713 (1954). — 4. KAPLAN, N. O., M. M. CIOTTI and F. E. STOLZENBACH, J. biol. Chemistry *221*, 833 (1956). — 5a. MCILWAIN, H. and R. RODNIGHT, Biochem. J. *44*, 470 (1949). — 5b. MCILWAIN, H. and R. RODNIGHT, Biochem. J. *45*, 337 (1949). — 6. KAPLAN, N. O., A. GOLDIN, ST. R. HUMPHREYS, M. M. CIOTTI and J. M. VENDITTI, Science, Washington *120*, 437 (1954). — 7. COPER, H. and H. HERKEN, Naunyn-Schmiedebergs Arch. exper. Path. *243*, 291 (1962). — 8. BRUNNEMANN, A., H. COPER and H. HERKEN, Naunyn-Schmiedebergs Arch. exper. Path. *245*, 541 (1963). — 9. LOWRY, O. H., N. R. ROBERTS and J. J. KAPPHAHN, J. biol. Chemistry *224*, 1047 (1957).

10. Burch, H. B., C. A. Storwick, R. L. Bicknell, H. C. Kung, L. G. Alejo, W. A. Everhardt, O. H. Lowry, C. G. King and O. A. Bessey, J. biol. Chemistry *212*, 897 (1955). — **11.** Herken, H., and V. Neuhoff, Hoppe-Seyler's Z. physiol. Chem. *331*, 85 (1963). — **12.** Neuhoff, V., Habilitationsschrift, Freie Universität Berlin (1964). — **13.** Klingenberg, M., Struktur und funktionelle Biochemie der Mitochondrien, II. Die funktionelle Biochemie der Mitochondrien. In: Funktionelle und morphologische Organisation der Zelle S. 69, Springer-Verlag Berlin, Göttingen, Heidelberg (1963). — **14.** Herken, H., Reduction of renal sodium and potassium transport following intracellular biosynthesis of abnormally structured nucleotides. Gastvorlesungen in den Pharmakologischen Instituten des Medical Center, Syracuse und der Harvard Medical School, Boston, (1965). — **15.** Herken, H., Drug-induced pathobiotic effects. In: Proc. 3rd. Pharmacological Meeting Vol. 4, p. 3, Pergamon Press Limited, Oxford, (1968). — **16.** Niklowitz, W. and I. J. Bak, Zschr. Zellforsch. *66*, 529 (1965). — **17.** Warburg, O. and W. Christian, Biochem. Z. *287*, 291 (1936). — **18.** Coper, H. and D. Neubert, J. Neurochem. *10*, 513 (1963). — **19.** Coper, H. and D. Neubert, Biochim. biophysica Acta, Amsterdam *89*, 23 (1964). — **20.** Neubert, D. and H. Coper, Biochem. Z. *341*, 485 (1965). — **21.** Herken, H. and R. Timmler, Naunyn-Schmiedebergs Arch. exper. Path. *250*, 293 (1965). — **22.** Brunnemann, A. and H. Coper, Naunyn-Schmiedebergs Arch. exper. Path. *246*, 493 (1964). — **23.** Kuschinsky, K. U., Enterale Resorption, Verteilung und Ausscheidung von Formaldehyd bei Ratten und Mäusen, Inaugural-Dissertation, Freie Universität Berlin (1965). — **24.** Gaitonde, M. K. and D. Richter, J. Neurochem. *13*, 1309 (1966). — **25a.** Beher, W. T., W. M. Holliday and O. H. Gaebler, J. biol. Chemistry *198*, 573 (1952). — **25b.** Beher, W. T. and W. L. Anthony, J. biol. Chemistry *203*, 895 (1953). — **25c.** Gaebler, O. H. and W. T. Beher, J. biol. Chemistry *215*, 527 (1955). — **25d.** Beher, W. T., G. D. Baker and M. Madoff, J. biol. Chemistry *234*, 2388 (1959). — **26.** Harris, Th. and V. Neuhoff, Naunyn-Schmiedebergs Arch. exper. Path. *253*, 221 (1966). — **27.** Neuhoff, V. and F. Köhler, Naunyn-Schmiedebergs Arch. exper. Path. *254*, 301 (1966). — **28.** Hicks, S. P., Amer. J. Path. *31*, 189 (1955). — **29.** Coggleshall, R. E. and P. D. McLean, Proc. Soc. Exp. Biol. Med. *98*, 687 (1958). — **30.** Coggleshall, R. E. and P. D. McLean, Transact. Amer. Neurol. Ass. *84*, 169 (1959). — **31.** McLean, P. D., in: Selective Vulnerability of the Brain in Hypoxaemia, p. 177 Blackwell Scientific Publication Oxford (1963). — **32.** Lierse, W., Zschr. Zellforsch. *67*, 86 (1965). — **33.** Peters, G., Personal Communication (1966). — **34.** Denk, H., M. Haider, W. Kovac and G. Studynka, Acta Neuropath. *10*, 34 (1968). — **35.** Neubert, D. and E. Oberdisse, Unpublished Experiments (1967). — **36.** Widnell, C. C. and J. R. Tata, Biochem. J. *92*, 313 (1964). — **37.** Blackstad, T. W., J. Comp. Neurol., Philadelphia *105*, 417 (1956). — **38.** Andersen, P., Acta physiol. Scand. *47*, 65 (1959).

Prof. Dr. H. Herken
1000 Berlin 33
Thielallee 69/73

Steroide und Haut

I. Mitteilung: Zur Bestimmung verschiedener C_{19}- und C_{18}-Steroide in Hauteluaten

Von L. Treiber und G. W. Oertel

Aus der Abteilung für Experimentelle Endokrinologie der Universitäts-Frauenklinik Mainz (Direktor: Prof. Dr. V. Friedberg)

(Eingegangen am 16. Januar 1968)

Zur Routine-Analyse verschiedener C_{19}- und C_{18}-Steroide in der Haut vom Menschen eluiert man 100 cm² der Hautoberfläche mit geeigneten Lösungsmitteln, unterwirft den Extrakt nach Entfettung einer Solvolyse und trennt neutrale und phenolische Steroide ab. In der neutralen Fraktion werden Dehydroepiandrosteron, Androsteron und Ätiocholanolon sowie Testosteron mit der 2,4-Dinitrophenylhydrazinmethode, in der phenolischen Fraktion die Östrogene durch Gaschromatographie ihrer Acetate quantitativ bestimmt. An Stelle der üblichen Konzentrationsangaben gibt das Konzentrationsverhältnis einzelner Verbindungen zueinander brauchbare Hinweise auf das Steroid-Muster der Haut.

Steroids and the skin. Communication No. 1

For the routine analysis of the different C_{19}- and C_{18}-steroids in human skin, 100 cm² of skin surface are eluted with suitable solvents, fats are removed from the extract, the steroids are redissolved, and the neutral and phenolic steroids are separated. In the neutral fraction, dehydroepiandrosterone, androsterone, aetiocholanolone and testosterone are quantitatively determined with 2,4-dinitrophenylhydrazine, and the oestrogens of the phenolic fraction by gas chromatography of their acetates.
In addition of the usual concentration data, the relative concentrations of the individual compounds are a useful indication of the skin steroid pattern.

Das Vorkommen von C_{19}-Steroiden in der menschlichen Haut ist seit geraumer Zeit bekannt (1—3). Desweiteren konnte im Hautgewebe ein Stoffwechsel derartiger Steroide nachgewiesen werden (4—6), wobei offenbar das im Kreislauf befindliche Dehydroepiandrosteronsulfatid (7) als Vorstufe der durch die Haut ausgeschiedenen C_{19}- und C_{18}-Steroide gelten dürfte (8).
Aufgrund dieser experimentellen Befunde erschien es angezeigt, eine Bestimmung verschiedener C_{19}- und C_{18}-Steroide in der Haut vorzunehmen, die gegebenenfalls auch zur Diagnose Steriod-abhängiger oder -beeinflußter Hautkrankheiten beitragen könnte.

Methodik

Gewinnung von Hauteluaten

Zur Gewinnung der in der Haut auftretenden freien Steroide, Steroidsulfatide, -sulfate (und -glucuronide) wird eine 10 × 10 cm große Hautfläche oberhalb des Knies mit 10 ml Äther-Äthanol-Wasser (1:4:1 v/v) und anschließend mit 10 ml Äthylacetat eluiert.