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Physiologia

EFFECT OF BLOOD PRESSURE CHANGES ON SINGLE UNIT ACTIVITY IN THE BULBAR RETICULAR FORMATION

By

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The effect of blood pressure changes induced in the anaesthetized cat has been studied on single unit activity in the bulbar reticular formation using extracellular microelectrodes. Blood pressure regulating reflexes were eliminated by sectioning the vagal and the sinus and glomus nerves. It has been found that a drop in blood pressure elicited by acetylcholine or by stimulating the central or peripheral stump of the vagus nerve usually increases, whereas a rise in blood pressure depresses, the various single unit activities. On the basis of the obtained results the specific activating effect of acetylcholine has been challenged and the increase in activity observed in response to acetylcholine-induced blood pressure decrease has been attributed to local tissue asphyxia. The depressing effect of an increase in blood pressure can be explained either by assuming that the cells are directly sensitive to blood pressure changes or by the presence in cerebral circulation of some unidentified pressure sensitive structure.

Bonvallet *et al.* (1954), as well as Dell (1960), Rothballer (1956), and Bradley and Mollica (1958) claimed that both acetylcholine and adrenaline are influencing the single unit activity in the mesencephalon and the medulla directly, *i.e.* not by inducing changes in blood pressure. On the basis of these findings Bradley and Mollica (1958) postulated that there are adrenergic and cholinergic mechanisms operating in the reticular formation. On the other hand, Baust and Katz (1961) and Baust and Niemczyk (1962) found that adrenaline does not directly affect single cell activity in these structures. Then Baust and Niemczyk (1963) and Baust *et al.* (1963) succeeded in showing that changes in blood pressure exert a direct depressing effect on single cell activity in the reticular formation, and they concluded that there must be some pressure sensitive neurones in the mesencephalic reticular formation, more precisely between the +5 and −5 frontal planes.

On the basis of earlier findings (Pórszász *et al.* 1961) we have concluded that cells of the bulbar reticular formation are directly sensitive to changes in blood pressure. Activity of these cells was usually inhibited by adrenaline and enhanced by acetylcholine. As single unit activity was augmented not

only by the latter agent but also by histamine and a variety of ganglion blocking agents, enhancement of single unit activity by acetylcholine is not a specific effect. The present paper deals with further observations in this field.

Methods

Experiments were made on a total of 26 cats anaesthetized intraperitoneally by 40 mg/kg of chloralose and 300 mg/kg of urethane. The head of the cat was fixed in a Horsley—Clarke stereotaxic device, and the cerebellum was exposed and removed by suction. The medulla was covered with cotton-wool soaked in warm 0.9% saline solution. Body temperature was kept constant by the aid of an infrared bulb placed about 80 cm above the animal. Blood pressure was monitored from the left femoral artery, and the contralateral femoral vein was used for injecting the substances. To avoid respiratory artefacts, 0.5 to 1.0 mg/kg of D-tubocurarine was given intravenously, a pneumothorax was made and artificial respiration was instituted. To eliminate circulatory and respiratory reflexes the carotid sinus and glomus nerves were dissected on both sides, and the vagus, depressor and sympathetic nerves were cut bilaterally (so-called deafferentated preparation).

The glass capillary microelectrodes with a tip diameter of 1 to 5 μ were filled with a 3 M solution of KCl (PÓRSZÁSZ and SZABÓ 1960) and introduced vertically into the appropriate points of the medulla by means of a device provided with a micrometer screw. The obex was used as the reference point and the distances were measured both in anterior (A) and lateral (L) directions from this point. To characterize the site of the electrode in the vertical direction, the surface was taken as reference (V_0). After the experiment, the exact sites of penetration of the electrodes were established on the surface of the medulla. The points where electrodes were introduced never exceeded 4 in number on one side, which facilitated evaluation and made the results reliable. In evaluating single unit activity, the criteria set up by BAUMGARTEN *et al.* (1954) were observed. As in the majority of cases the electrodes lay extracellularly, not only single unit activity but also the activity of several neurones was recorded. It was usually possible to distinguish single unit activity from that of more cells, for the latter exhibited different amplitudes, eventually also different frequencies, in the same record. Most records presented in this paper show the activity of 2 or 3 units. As the indifferent point for the microelectrode a copper plate of 6 sq.cm surface was placed under the skin of the back. For stimulation a square-wave pulse generator was used which allowed amplitude, duration and frequency of the stimulus to be altered independently. To avoid inrush of external stimuli, a high-frequency isolation unit was employed (PÓRSZÁSZ and SZABÓ 1959). Action potentials were amplified by a R.C.-coupled differential amplifier with a high input resistance, and recorded on a DISA dual-beam oscilloscope. Unit activity was simultaneously recorded on tape, which greatly facilitated the evaluation of changes observed in electrical activity (PÓRSZÁSZ and SZABÓ 1959).

Results

The activity of a total of 93 cells was recorded, the effect of blood pressure changes could be studied in 45; in the other cells activity was not resumed after the change in blood pressure and had therefore to be neglected. *Fig. 1* shows the medullary co-ordinates from where the potentials were recorded. The "functional" distribution of the neurones is shown in *Table I*, so-called steady activity was recorded in 40.8% of the cases. These units exhibited a 10 to 20 c/s uniform activity which was almost regular throughout the records; it was not affected by the stimulation of somatic afferent nerves. Inspiratory and somatic neurones occurred in equal numbers, approximately 14%. There was a considerable convergence on these latter cells particularly from the trigeminal and

Table I

Effect of acetylcholine, adrenaline, and vagal stimulation on discharge activity of cells in the bulbar reticular formation

Type of identified neurones	Number of neurones	Incidence per cent	Adrenaline	Acetylcholine	Vagal stimulation
Steady activity	36	40.8	−8 +2 ⌀3	−1 +13 ⌀2	−1 +5 ⌀1
Inspiratory	13	14.0	−1 ⌀1	+1 ⌀1	
Expiratory	2	2			
Synchronous with pulse	8	8.6	−1	+2	+3*
Somatic	13	14.0	+1	+4 ⌀1	⌀1
Burst activity	16	17.2	−4 +1 ⌀1	+6	+5 ⌀3
Other: vasomotor neurones	3	3	−3	+3 +3**	
Total:	91	100	−17 +5 ⌀5	−1 +32 ⌀4	−1 +13 ⌀5

Symbols: + Discharge frequency increased in response to the experimental procedure
− Discharge frequency reduced
⌀ No change in discharge frequency
* In one experiment the peripheral vagal stump was stimulated
** Cessation of artificial respiration increased the activity in 3 cases

limb areas, and the incidence of cells showing periodic burst activity which, however, could not be activated somatically, was 17%. These latter were certainly identical with the cells described by Moruzzi (1954) in the medioventral bulbar reticular formation. Other activities occurred considerably less frequently.

Changes in blood pressure were elicited by intravenous adrenaline or acetylcholine, and by stimulating the peripheral or central stump of the divided vagus nerve. In some of the experiments, adrenaline and acetylcholine were injected into the common carotid after the branches of the external carotid artery had been ligated. Not all single units were studied under all the experimental conditions mentioned above. The obtained results have been

summarized in *Table I.* It is evident from this Table that adrenaline usually depressed, whereas acetylcholine enhanced, the electrical activity of the cells of the bulbar reticular formation. Stimulation of the central or peripheral vagal stump enhanced single unit activity.

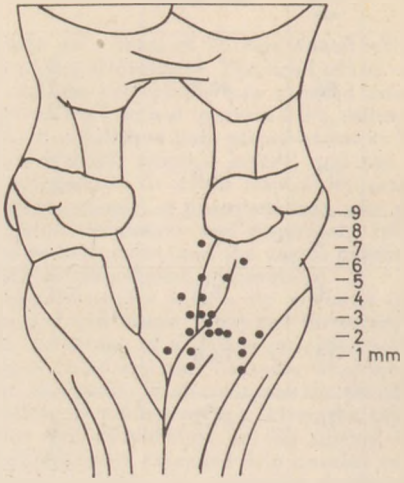

Fig. 1. Location in the medulla of the recorded cell activities

In the following, several characteristic changes in activity, apparently determined functionally, will be described in detail.

A. Steady activity

Fig. 2 shows the activity of 3 different neurones; the deflections differ in amplitude but every activity displays a steady frequency. The co-ordinates of the electrode used to obtain this record were $A_2L_3V_{1,000}$ μ. Tracing *a* shows the effect of stimulation of the central vagal stump with the parameters 10 c/s, 10 msec, 10 V. The frequency of cell activity increased from 11 to 14 c/s in response to this stimulus, while blood pressure was reduced from 120 mm Hg to 90 mm Hg. As blood pressure rose after cessation of the vagal stimulation, the frequency of unit activity decreased below the initial value.

Fig. 3 demonstrates the activity of the same neurone population in response to 1 μg/kg of intravenous acetylcholine. Blood pressure was more markedly reduced than by vagal stimulation (from 120 mm Hg to 60 mm Hg), and the frequency of unit activity was also more increased, from 8 to 9 c/s to 13 to 14 c/s. With the rise in blood pressure, activity was successively inhibited, disappearing fully after a certain time (trace *b*) and then reappearing at low frequency (trace *c*).

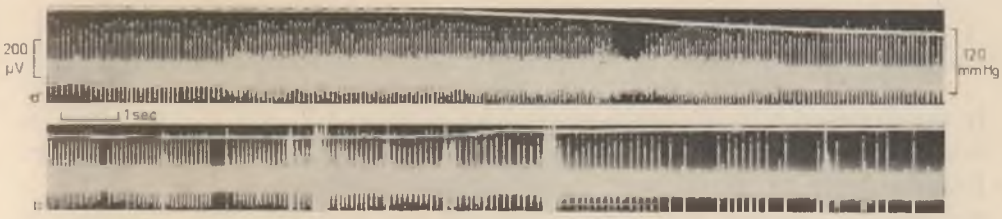

Fig. 2. Cat No. 282. Anaesthesia was induced by chloralose (40 mg/kg) and urethane (300 mg/kg) given intraperitoneally. Effect of vagal central stump stimulation on blood pressure (upper tracing) and discharge activity of a neurone population showing a steady activity at rest. Three cell activities with different amplitudes are clearly distinguishable

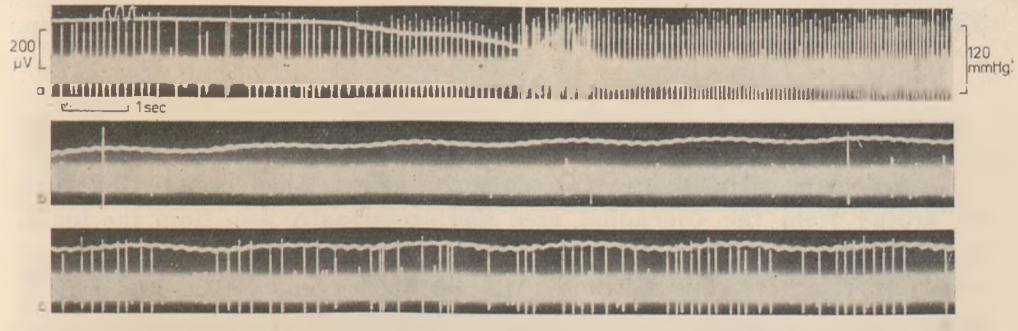

Fig. 3. Cat No. 282. Effect of 1 μg/kg of acetylcholine on blood pressure (upper tracing) and on the discharge activity of the neurone population with steady activity (lower tracing). Three different unit activities can be distinguished. *a:* Blood pressure falls in response to 1 μg/kg acetylcholine, activity is increased. *b:* The rise in blood pressure causes a transient inhibition of spike activity. *c:* Activity reappears

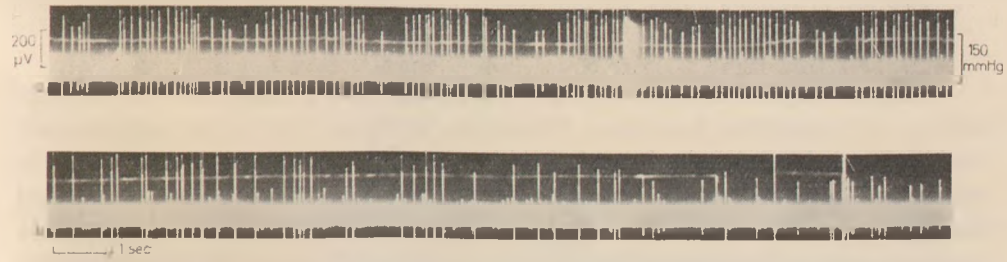

Fig. 4. Cat No. 282. Effect of 5 μg/kg of adrenaline intravenously on the activity of the same neurone population as shown in *Figs 2* and *3*. Upper tracing, blood pressure. Lower tracing, discharge activity. *a:* Initial activity. *b:* The frequency of spikes is reduced in response to adrenaline

Fig. 4 shows the activity of still the same neurone population. The intravenous administration of 5 μg/kg of adrenaline markedly depressed the frequency of spontaneous activity, concomitantly with a sustained rise in blood pressure.

B. Neurones displaying an activity synchronous with the pulse

Of the 93 neurones studied only 8 showed an activity which was completely synchronous with the heart revolutions. Tracing *a* in *Fig. 5* shows such an activity. In this record, usually two bursts appeared after each heart beat. Unfortunately, the respiratory waves could not be included in the record; it is nevertheless apparent that corresponding to the inspirations the frequency of bursts per pulse wave increased periodically and the activity became somewhat less synchronous. Tracing *b* shows the activity after the injection of acetylcholine; periodicity of the activity disappeared almost completely. After the effect of acetylcholine had subsided, initial activity could again be recorded. This activity was enhanced also by stimulation of the peripheral vagal stump.

C. Somatic neurones

We succeeded in isolating 13 neurones which could be activated by stimuli arising from the trigeminal area and occasionally also from the limbs. Figure 6 (tracing *a*) shows such an activity; the co-ordinates of the recording site were $A_3L_4V_{3,000}$ μ. Gentile patting of the nose caused large action potentials to appear, after which activity was depressed. These neurones seem to be identical with those described by MORUZZI (1954). Activity of this unit was considerably enhanced by acetylcholine (tracing *b*), and the period of inhibition elicited by patting of the nose was much shorter in duration.

D. Vasomotor neurones

An attempt has been made to reveal single units playing a role in the regulation of vasomotor functions. Therefore, artificial respiration was stopped for brief periods of time in several experiments. It has been postulated that cells which respond to asphyxia with an increase in activity but cannot be classified among any of the cells listed in *Table I* in which the different categories have been set up according to functional characteristics, may have a role in the regulation of blood pressure. Three units with the co-ordinates: $A_1L_3V_{3,800}$, $V_{3,850}$ and $V_{3,950}$ μ, respectively, were revealed which may have been involved in the maintenance of vasomotor tone. These neurones exhibited a steady activity, which was considerably augmented 30 to 40 sec after cessation of artificial respiration. The rise in blood pressure occurred only after the enhancement of single unit activity. As blood pressure rose, activity was depressed. The protocol of one of these experiments is presented here to demonstrate the changes in the electrical activity of cells presumably having blood pressure regulating activity.

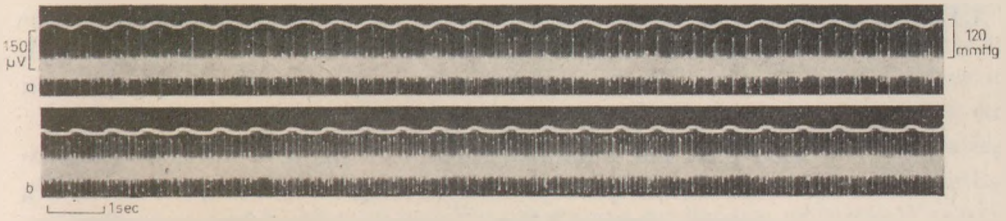

Fig. 5. Cat No. 281. Activity is synchronous with the heart revolutions. Effect of intravenous acetylcholine (1 μg/kg) on blood pressure (upper tracing) and discharge activity (lower tracing). The record is made up of the spike activities of two neurones. *a:* Activity prior to acetylcholine. *b:* Effect of acetylcholine

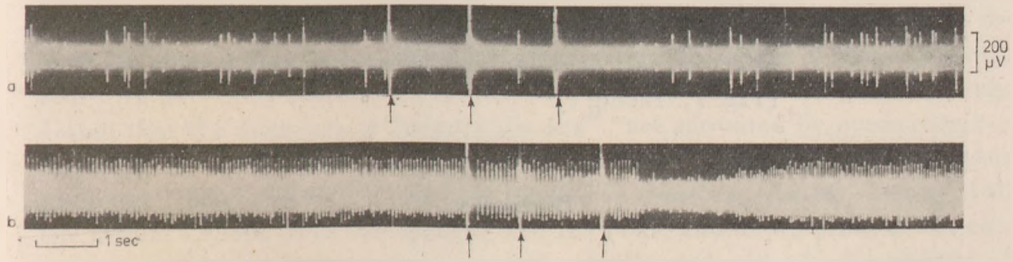

Fig. 6. Cat No. 283. The microelectrode is located in the nucleus of the spinal tract of the trigeminal nerve (co-ordinates: $A_5L_1V_{3,000}\,\mu$). Neurone with irregular activity which could be inhibited by stimulation of the trigeminal receptor area. Activity of this unit was enhanced by 1 μg/kg of intravenous adrenaline; the duration of inhibition after trigeminal stimulation became shorter after acetylcholine. Arrows indicate gentile patting of the nose

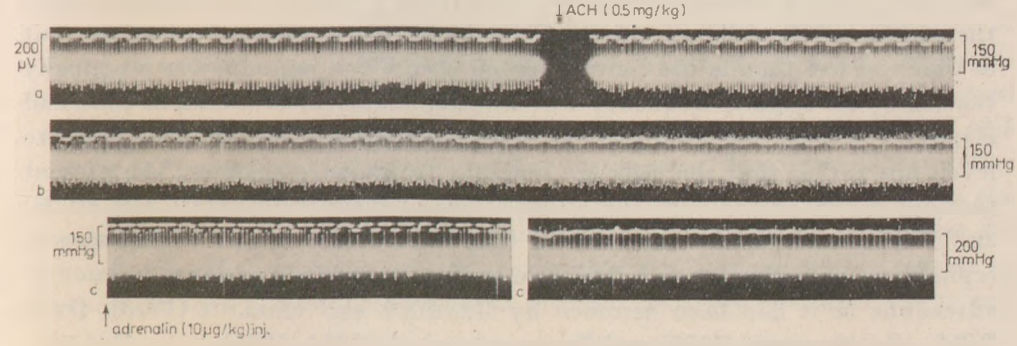

Fig. 7. Cat No. 289. Effect of acetylcholine (0.5 μg/kg) and adrenaline (10 μg/kg) given intravenously on the discharge activity of a "vasomotor unit". Upper tracing, blood pressure. Lower tracing, spike activity. *a, b:* Effect of acetylcholine. *c:* Effect of adrenaline

$A_1L_3V_{3,800}$ μ

3.00 p.m. Steady activity with regular high-amplitude deflections. The intravenous administration of 0.5 μg/kg of acetylcholine is followed after 20 sec by a considerable increase in frequency (*Fig. 7*, tracings *a* and *b*).

3.03 p.m. The administration of 10 μg/kg of adrenaline diminishes the frequency. At a blood pressure level of 140 mm Hg activity ceases (*Fig. 7*, tracing *c*), then reappears at 80 mm Hg.

3.24 p.m. Artificial respiration is stopped. After 30 sec, the frequency begins to increase prior to the change in blood pressure. After 60 sec the respiration is reinstituted; this is followed by a rapid rise in blood pressure and the complete disappearance of unit activity.

3.28 p.m. The frequency observed prior to asphyxia has been regained. The neurone showed an activity till 3.38 p.m.; the effect of acetylcholine and adrenaline could again be tested (*Fig. 7*, tracing *c*).

Discussion

The presented results indicate that there are cells in the bulbar reticular formation which respond directly to the elevation of blood pressure. We have found that in the cat which has been deprived of all the known pressure sensitive afferent systems, adrenaline inhibited the function of these cells. One of the most impressive findings can be seen in *Figs 2* and *3;* it is evident from these records that it was not the adrenaline but the rise in blood pressure which inhibited unit activity. Stimulation of the central vagal stump and the administration of acetylcholine resulted in a drop in blood pressure and an increase in activity; when subsequently blood pressure had returned to its initial value, the activity of the cells was promptly inhibited. The number of spikes per unit of time was less than in the control period when blood pressure was practically the same; the original frequency was reattained only after a certain time had elapsed. It seems, thus, that activity is not inhibited at a certain blood pressure level, but rather that the cells are sensitive to pressure changes. The present findings are in accord with the observations of Baust and Niemczyk (1963) and Baust *et al.* (1963) who claimed that the activity of cells in the mesencephalic reticular formation is inhibited by the rise in blood pressure and not by adrenaline as it had been assumed by Bradley and Mollica (1958), Dell (1960), Rothballer (1956) and Bonvallet *et al.* (1954, 1956).

The observed response to acetylcholine cannot be attributed to the direct specific effect of the compound, since it cannot penetrate the blood-brain

barrier. The possibility that acetylcholine exerted a specific stimulating effect on the neurones of the CNS could be excluded also on the basis of its failure to exert a greater effect on unit activity when injected into the carotid artery in the same dose as that administered intravenously. In both cases, activity did not increase before the diminution of blood pressure. The specific activating effect of acetylcholine was challenged also by the finding that the stimulation of both the central and peripheral vagal stumps resulted in an increase of unit activity, concomitantly with the drop in blood pressure.

SALMOIRAGHI (1962) studying the activity of bulbar cells the function of which is affected by changes in blood pressure, claimed that these cells may play a role in various vasomotor functions. He was able to isolate two types of such cells. In "population one" he found among 53 a total of 12 cells which exhibited a decrease in activity in response to a drop in blood pressure, whereas activity was enhanced by the rise of blood pressure. The second type ("population two"), 41 cells of 53, thus the majority of the observed population, responded with an increase in spike frequency to the reduction of blood pressure and with depressed activity to the increase in blood pressure. The author suggested that the neurones of "population one" "are activated by pressoreceptor discharge", whereas those of "population two" "are inhibited by pressoreceptor discharge". The majority of the cells studied in these experiments belonged to the medial structures of the medulla and the pons, and deprivation of the animal from its pressoreceptor structures greatly reduced the possibility of finding such cardiovascular neurones. After division of the sinus and of the vagus nerves, only 5 cells could be revealed which retained their sensitivity to blood pressure changes. In view of these findings it seems probable that the neurones studied by SALMOIRAGHI *were in most part not true vasomotor neurones, but rather cells which participated in certain vasomotor reflexes.*

The present results indicate, however, that changes in blood pressure may affect the activity of a great variety of CNS cells. The most likely explanation of the activating effect of blood pressure fall seems to be the deterioration of tissue metabolism, the difficulties in adequate oxygen supply and carbon dioxide transport which lead to local tissue asphyxia. Depending on the functional state of the cell, the discharge activity of the neurone either increases, and this occurs in the majority of cases; in other instances it does not affect activity throughout longer periods of time, or blocks it completely. This explains the variability of responses given to acetylcholine, as the drop in blood pressure did not always enhance spike activity, and also the finding that cessation of respiration for 1 min either augmented, or inhibited or did not affect, single unit activity.

As to the depressing effect of the increase in blood pressure we share the view of BAUST (1963) who claimed that either the cells are directly susceptible

to the rise in pressure or there are some unidentified pressoreceptor structures in the cerebral vascular bed.

Summing up, it seems that cells located in the so-called inhibitory (or depressor) area of the bulbar reticular formation and in the adjacent lateral structures are extremely sensitive to blood pressure changes, responding with considerable alterations in discharge activity. As this area of the CNS is of vital importance, the observed reactions may play a significant role in homeostasis and, in the deafferentated animal, constitute the last link in the chain of protective reactions.

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THE RELATIONSHIP OF THRESHOLD AND OPERATING LEVEL IN THE HUMAN OBSERVER'S RESPONSES TO ACOUSTIC STIMULI

By

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By a complex recording of EMG, EEG and GBR reactions, the relation between the threshold of the analyzer and the various levels of operation have been studied. A sequential analysis of the data obtained allowed the following conclusions.

1. The latency of EMG reactions depends on the intensity of the external actual stimulus kept in memory and its nearness to the standard intensity of stimulation serving as the reference pattern. At the same time, the after-effect of EMG is determined exclusively by the intensity of the stimulus.

2. The latency of the EEG and GBR response is presumably determined exclusively by the different summation periods of the "on" and "off" neuronal elements.

3. The appearance of the EEG and GBR responses, as effects regulating the components of the orientation reaction, depends on the physical force of the stimulus and on those processes which compare the actual stimulus with the standard stimulus pattern kept in memory.

4. The mechanism of erroneous excitatory responses is to be sought presumably in the "own noise" of the analyzer, as well as in fluctuations in the sensitivity of the levels of operation.

5. The appearance of EEG and GBR reactions independently from the standard stimulus pattern in the memory suggests that in addition to the various levels of operation serving to carry out motor reactions the analyzer possesses a constant threshold.

G. T. Fechner (1860) establishing his metric principle of sensitivity was well aware of the difficulties in estimating the absolute threshold; they mainly centre around the problem of defining the beginning point of a sensation. Still now the general view is that we must consider the methods in determining threshold as an uncontrolled variable (Corso 1963). Moreover, the threshold estimated by conventional procedures can only be a global indicator of the observer's hit rate and does not permit the analysis of all the contingencies of a stimulus-response situation, *e.g.* it does not convey any information concerning the observer's operating characteristics. Offering a more suitable approach for eliminating the methodological insufficiencies, the statistical decision theory has gained a wide field of application for studying the sensitivity of human analyser systems (Peterson *et al.* 1954; Swets *et al.* 1961; Atkinson 1963; Price 1966; Green and Swets 1966). This theory provides a method which permits the separation of the observer's response-criterion and his sensitivity

(TANNER and SWETS 1954). In the process of signal detection the theory attaches a basic significance to the criterion or "operating level" (POLLACK 1961; SOKOLOV 1964) used by the observer in relating unknown signals to the categories of "noise" or "signal + noise". A further development of this theory has led to the negative concept of threshold (SWETS 1961); a tendency to substitute the threshold concept by the concept of operating level has appeared. There arises the question, to what extent this way of identification is justified.

The statistical decision theory is mainly concerned with the momentary intensity value of a signal. It is less suitable, however, to describe the perception of signals consisting of a succession of instantaneous values. In this case it seems to be more favourable to apply the sequential analysis of WALD (1947), where the time, required for the detection of a signal and representing a component of latent period in the response, has become the most important parameter of the observer's reaction. Using this way of approximation in her earlier work, KORZH (1963) studied the relationship of threshold and operating level in the responses to light stimuli of the human observer. In the present work the investigation is extended to the acoustic area. For the purpose of an analytic study of this relationship three methods have been applied in combination. *1.* Complex registration of voluntary (motor) and involuntary (autonomic) reactions (which allowed to delimit responses depending on the operating level from those not connected with it directly. *2.* Method of "continuous tracking of the stimulus" (SOKOLOV 1964). This allows to study within one experiment the transition from a situation of "noise" to a situation of "signal + noise", as well as the reverse of it. *3.* Introduction of a fixed standard stimulus pattern (SSP), which gave a possibility for the direct regulation of the operating level, thus avoiding the application of indirect methods such as the variation of reward and punishment.

The present study was undertaken to test the following questions. *(a)* How do the latent period and the after-effect in EMG, EEG and GSR depend on the intensity of sound stimuli at different operating levels? *(b)* How does the hit and false alarm probability of EMG, EEG and GSR depend on changes in intensity of sound stimuli at various operating levels?

Methods

A total of 58 experiments was carried out on 12 adult subjects of 20—24 years of age. In the first series, the natural development of the operating level was studied. The subject, sitting comfortably in a deck-chair in a sound-proof room, was given the instruction: "When you hear a sound you must immediately clench your fist and keep it clenched until the sound has stopped."

In the second series the observer's response characteristics were tested at fixed operating level. This meant a SSP, which was to be kept in mind and served as a criterion concerning the fulfilment of a correct response. The following instruction was given: "Remember well the presented sound but respond by clenching your fist only if you hear a sound agreeing with it in intensity or louder. Do not respond if the sound is weaker."

A sound of 500 c/sec was used as signal, presented by means of a sonic generator type *ZG-10*. Sound intensity was varied between —20 db and 60 db beyond the threshold of audibility; 6—7 sound signals of different intensity were applied within that range. For the purpose of producing an operating level, a SSP was presented ten times before beginning of the experiment to ensure that the SSP would be remembered well. Each intensity value turned up ten times during one session. Three sounds of different intensity were introduced as SSPs: 40, 20 and 10 db beyond the threshold of audibility but in one series the characteristics of only one operating level were studied. Sound intensity at which the subject reported "hearing" 50% of the time was considered the threshold of sound detectability.

Responses serving as indicators (EMG from the right hand, EEG in bilateral occipito-parietal bipolar leads, and GSR according to TARCHANOFF's method) were continuously registered by means of an eight-channel SANEI EEG apparatus.

The method of "continuous tracking of the stimulus" has made it possible to analyse the responses, in contrast with the classical psychophysical experiments, not only in the moment of switching on a stimulus, but during its whole duration, after having switched it off, and also in the intervals between stimuli. Consequently two states of a stimulus were possible: the presence of a stimulus, indicated by (i), and its absence (i_0). The EEG recording of the whole experiment was divided into equal segments corresponding to the 5 sec duration of the stimulus. Each segment was analysed according to the presence (j) and absence (j_0) of the response. The relationship of stimulus and response was characterized by a conditional probability.

$P_{j/i}$ = probability of the presence of responses under condition of the presence of a stimulus (hit probability)

P_{j/i_0} = probability of the appearance of responses under condition of the absence of a stimulus (false alarm probability)

As the criterion of an EEG response was considered the blockade of alpha rhythm for 1 sec or longer and the diminution of its amplitude to one third of the background EEG. EMG activity lasting not less than 0.2 sec and reaching at least 15 mV in amplitude served as the criterion of an EMG reaction. In addition, the latency and after-effect (presence of a reaction after switching off the stimulus) of EEG and EMG responses as well as the latent period and amplitude of GSR were estimated.

The arithmetical mean of the mentioned response parameters was calculated for each subject as well as for the whole experimental group. The hit probability of EMG responses with long and short after-effect was also defined using the formula by SOKOLOV and MICHAILEVSKAIA (1961); 0.8 sec or longer after-effects were regarded as long ones, while those of 0.1—0.4 sec, as short ones.

Results

I. Dependence upon stimulus intensity of the latent period and the after-effect of EMG responses

In the first series, where SSP was not introduced, it was found that parallel to the reduction of stimulus intensity the latent period and the after-effect of EMG responses increased. It agrees well with data gained earlier in experiments on the absolute threshold of acoustic analyser by means of the "continuous stimuls tracking" method (SOKOLOV 1958; KORZH 1963). In the case of strong stimuli the EMG latencies were found to be shorter than the after-effect, while with weak signals the opposite was observed *(Fig. 1)*.

By means of a more detailed analysis of results it was possible to distinguish two groups of four persons each. In the first group the after-effect changed slightly on reducing the stimulus intensity (0.5 sec with strong sounds and 0.8

sec with weak ones, on the average), while in the second group a greater change was characteristic (0.4—0.5 sec with strong sounds and 1—1.5 sec with weak ones).

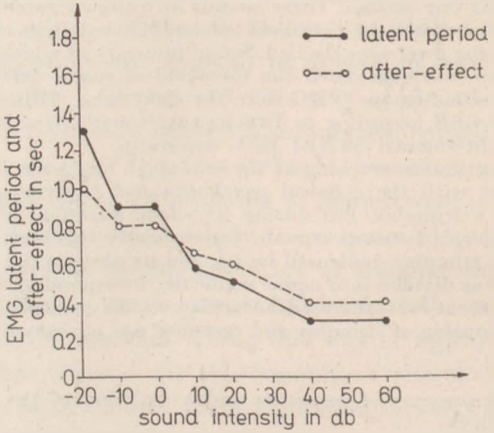

Fig. 1. Dependence on the stimulus intensity of the latent period and the after-effect of EMG responses (group mean)

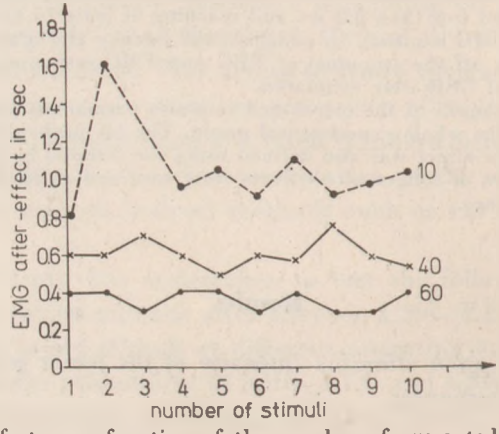

Fig. 2. EMG after-effect as a function of the number of presented stimuli. The different curves correspond to sound intensities of 10, 40 and 60 db (group mean)

The question arises what may cause the shortening of the EMG after-effect as compared to the latent period. Presumably it is connected with a conditioned reflex elaborated on time (on the 5 sec stimulus duration) and it enables the subject to prepare himself for stopping the motor response. This supposition, however, involves that the duration of the after-effect must be a function of the number of stimuli. A diagram of the after-effect-dynamics *(Fig. 2)* illustrates that it cannot be the case, because the duration of the after-effect

does not vary with the number of trials; it remains unchanged at a definite level depending on the stimulus intensity. A further analysis of the findings demonstrates how the hit probability of EMG responses with long and short after-

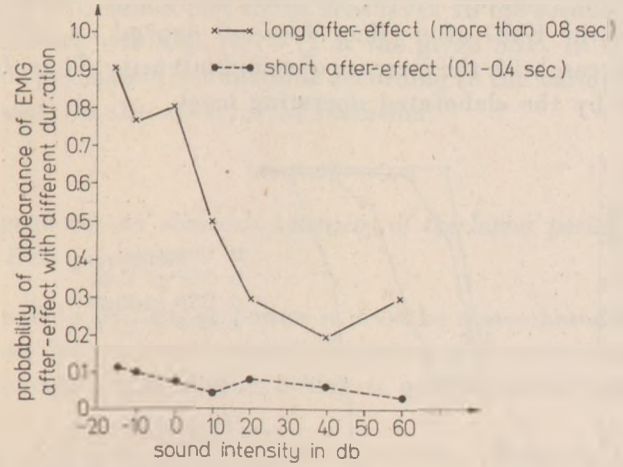

Fig. 3. Probability of appearance of EMG after-effects of short and long duration and their dependence on stimulus intensity

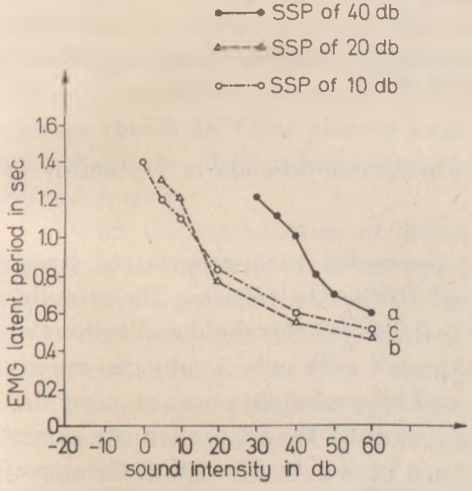

Fig. 4. Dependence on stimulus intensity of EMG latent period at different standard stimulus pattern (SSP) values.

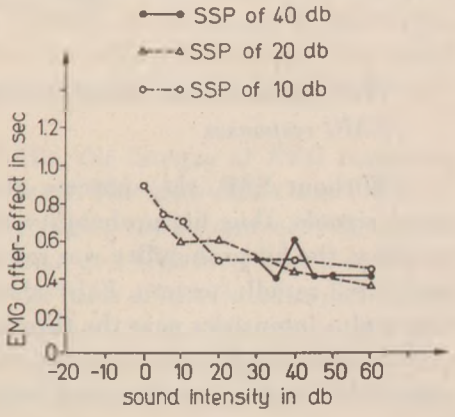

Fig. 5. Dependence on stimulus intensity of EMG after-effects at different SSP values (group mean)

effects, depends on stimulus intensity. It can be seen in *Fig. 3* that the hit probability of responses with long after-effect increased parallel with the reduction of sound intensity, especially in the threshold zone. At the same time the hit probability of an EMG with short after-effect was very low.

In the second series, where SSP was introduced, it was found that the EMG latencies ceased to be submitted to the so-called "power law". *Fig. 4* shows that the latent period of EMG increased on strong sound signals with high intensity SSP (diagram *a*). On the other hand, *Fig. 5* makes it clear that the duration of the EMG after-effect does not depend on SSP and shows a tendency to decrease with the increase of sound intensity. Thus, EMG latencies are determined by the elaborated operating level.

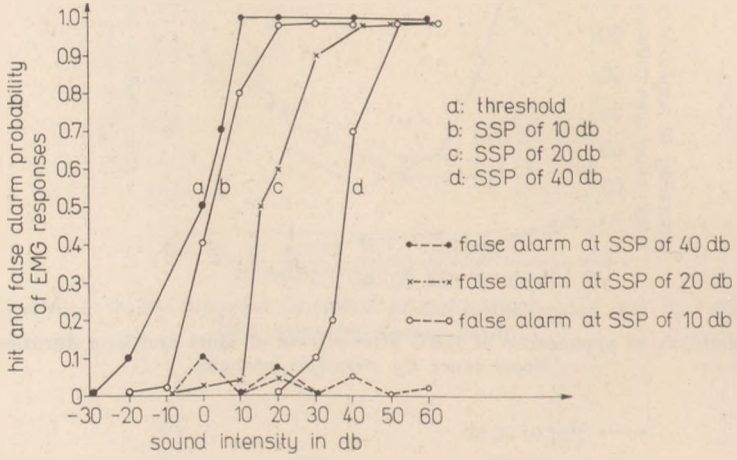

Fig. 6. EMG hit and false alarm probability as a function of stimulus intensity at different SSP values (group mean)

II. Dependence on stimulus intensity of hit and false alarm probability of EMG responses

Without SSP, the subjects always responded to appropriately strong sound signals, thus hit probability reached 100%. On reducing the stimulus intensity, the hit probability was reduced to 0.5 at the threshold and below this level it fell rapidly to zero. False alarm appeared with only 5 subjects, mainly at stimulus intensities near the threshold, and hit probability was at most 0.1.

In the second series, according to the given SSP the hit probability curves were shifted as compared to each other. It can be seen in *Fig. 6* that the curves have an approximately sigmoid shape. If SSP fell in the threshold zone (at 10 db), its psychometric function coincided roughly with the curve plotted on the basis of the first experiment, *i.e.* with the threshold. The next SSP (at 20 db) gave a curve shifting 5 db towards the sounds of higher intensity. With an SSP at 40 db beyond threshold, the curves shifted in such a manner that 50% probability corresponded to 37 db, *i.e.* the threshold nearly coincided with the operating level. False alarm with a probability value between 0.05 and 0.1 appeared with stimuli near SSPs, independent of their absolute intensity.

According to the accuracy of SSP retention the subjects were divided into two groups. In the first group of 5 persons, a significant increase in the operating level could be observed as compared to the SSP. The hit probability at SSP abruptly fell, sometimes to the zero level. In the second group of 5 persons, hit probability was high (0.7—1) at the given SSP. In our opinion, the sigmoid form of psychometric functions according to the various SSPs was due to the uncertainty in the accuracy of retention.

III. Dependence on stimulus intensity of the latent period and the after-effect of EEG responses

In the analysis of EEG responses it must be remembered that the blockade of alpha rhythm on sound stimuli occurring in occipital leads is to be regarded as an unspecific activation which is quickly extinguished in healthy subjects.

In good agreement with earlier observations (Gersuni 1955; Sokolov 1958) it was found that the latency of EEG responses increased on reducing sound intensity. Its after-effect, however, decreased at sounds of medium intensity and increased in the zone of strong stimuli. An increased orienting reflex activation was characteristic of the threshold zone which manifested itself with a prolonged alpha rhythm depression. This might be explained by the more difficult conditions for signal detection. Stimuli of an intensity below the average threshold value elicited weak EEG desynchronization and this could not reliably be distinguished from the spontaneous fluctuations of the background activity.

With the introduction of different SSPs, the latency of EEG responses proved to be independent of the operating level. The after-effect was found to appear with a maximum value at stimuli belonging to the zone of SSP, except at the threshold zone (10 db), where it remained at a high level over a considerable intensity range (See *Figs 7* and *8*). On the basis of individual experimental data, two groups of subjects could be distinguished. In the first group of 7 persons, on the background of the partially extinguished orienting reflex the alpha rhythm blockade appeared only at the very instant of stimulus presentation. It manifested itself with an increase of the after-effect to SSP and intensities near to it. In these cases the latent period was usually short. In the second group of 5 persons, latency and after-effect of the EEG varied in parallel.

From the above it has been concluded that the latency of EEG responses does not depend on the given operating level. On the other hand, the strongest after-effect was caused by stimuli close to the given SSP. This might have been due to a more difficult differentiation of signals, but also to the effect of stronger stimuli.

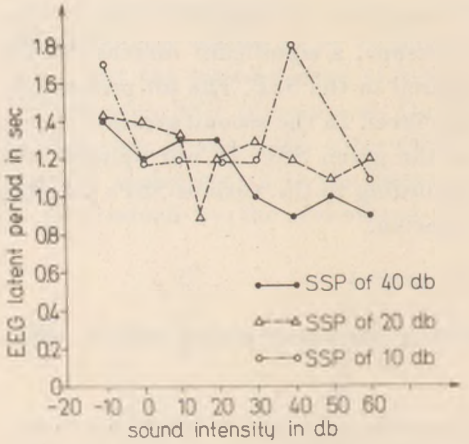

Fig. 7. Dependence on stimulus intensity of EEG latencies at different SSP values (group mean)

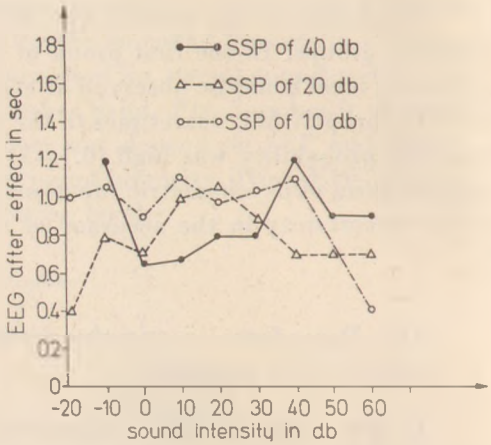

Fig. 8. Dependence on stimulus intensity of EEG after-effects at different SSP values (group mean)

IV. Dependence on stimulus intensity of the hit and the false alarm probability of the EEG

The hit probability of EEG responses was higher with threshold than with strong sounds if SSPs had not been introduced, but it never reached the value of 1. There were few false alarms with the majority of subjects. These characteristics of the EEG responses are connected with the extinguishing process of the orienting reflex (*Figs 12, 13* and *14*).

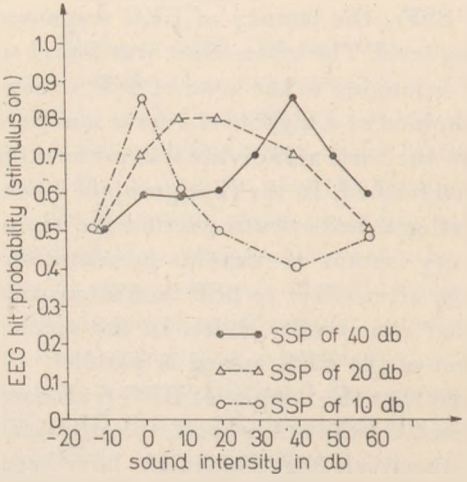

Fig. 9. Hit probability of EEG responses to stimulus "on" at different SSP values (group mean)

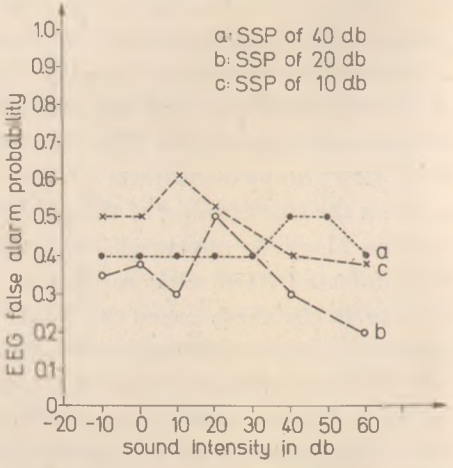

Fig. 10. EEG false alarm probability at different SSPs (group means)

Concerning SSPs of different intensity, hit probability was at its maximum in the zone of a given SSP. False alarm appeared with intensities near to the SSP, but in the case of 40 db SSP it often shifted towards strong sounds (*Figs 9* and *10*). Since alpha rhythm blockade as an unspecific activation was subjected to rapid extinction, it was necessary to define the hit probability of

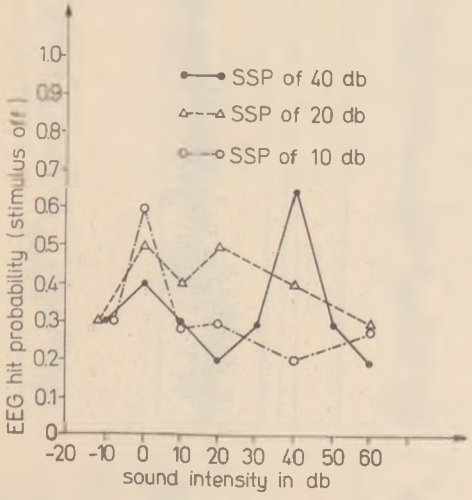

Fig. 11. Hit probability of EEG responses to a stimulus "off" at different SSPs (group mean)

the after-effect occurring with the switching off the signal for each SSP. It was found that the hit probability for after-effects reached its maximum for the zone of a given SSP *(Fig. 11)*. On the basis of individual curves of hit and false alarm probability, the shape of curves is not due to a divergence inside the group, but represents first of all the peculiarity of EEG activation.

Comparing the change of hit and false alarm probabilities from experiment to experiment, the phenomenon of extinction manifested itself especially in the diminution of false alarm probability, but slightly in the hit probability rate as well. This level slowly decreased, becoming eventually equal for all sound intensities.

V. Dependence on stimulus intensity of GSR amplitude and latency

Only 7 subjects' data were analysed here, because of the rapid GSR extinction in the 5 persons.

It was found that GSR had a short latent period for strong sounds while parallel with the reduction of stimulus intensity — especially near the threshold

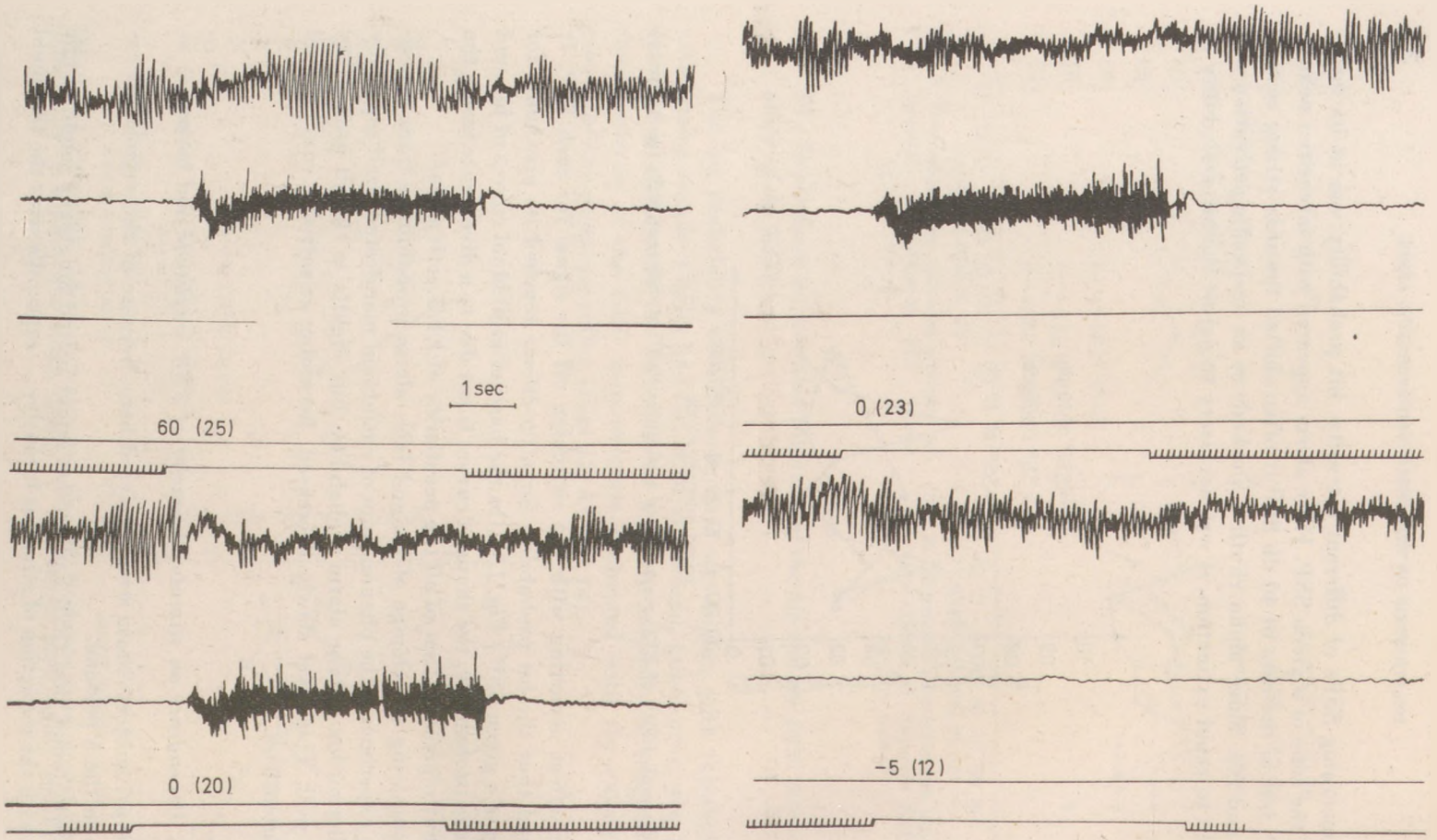

Fig. 12. Extinction of EEG alpha rhythm depression by strong and weak sound stimuli as a function of the number of presented signals in the first series of experiment. The top curve shows the EEG from bipolar occipital leads. The second is the EMG, the third curve the GSR. The bottom curve indicates the stimulus marking. The first numeral designates the sound intensity in db beyond the threshold of audibility. The second numeral in brackets is the number of trials

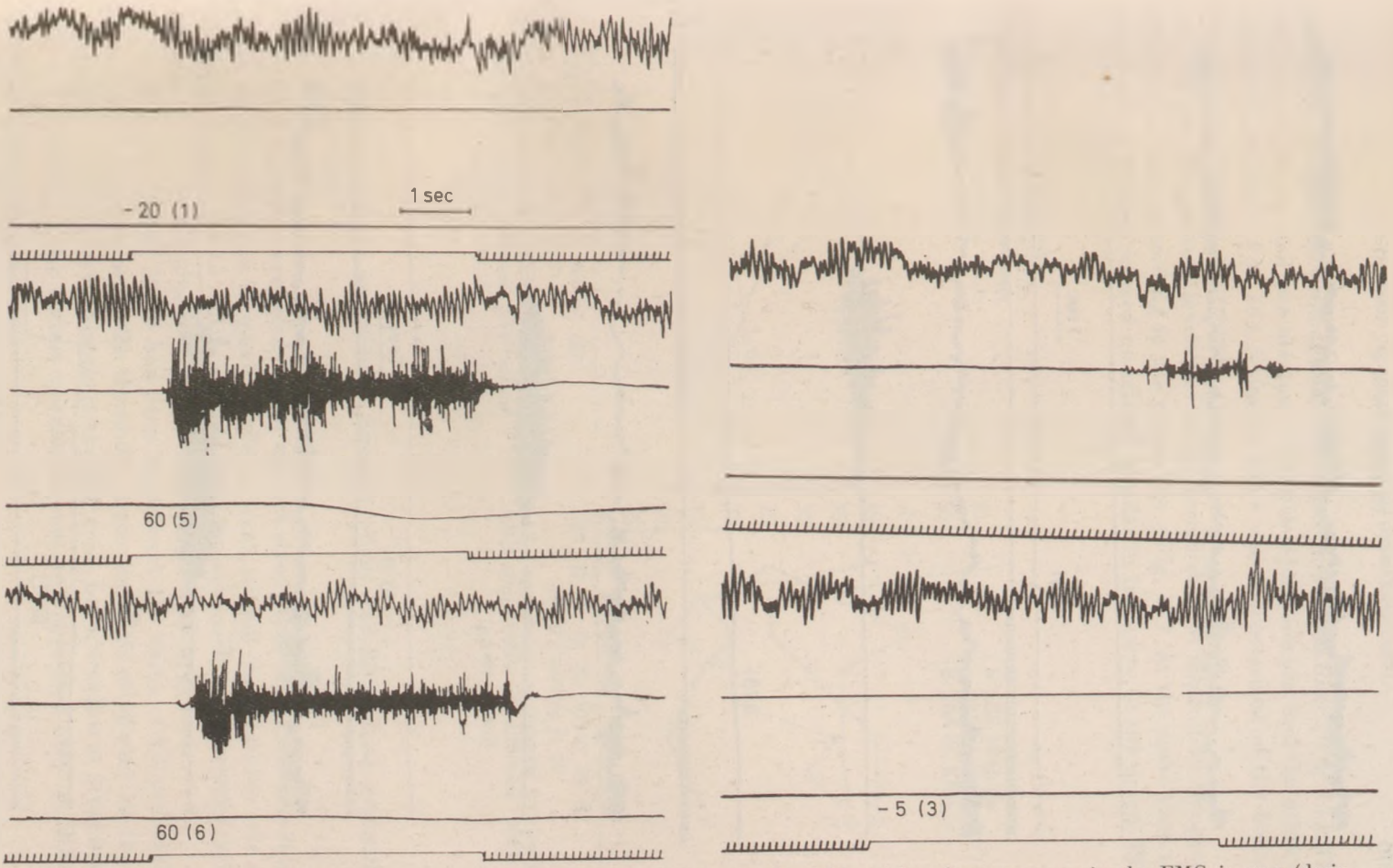

Fig. 13. Extinction of EEG and GSR activation during successive trials. On the right, a false alarm response in the EMG is seen (designation as in *Fig. 12*)

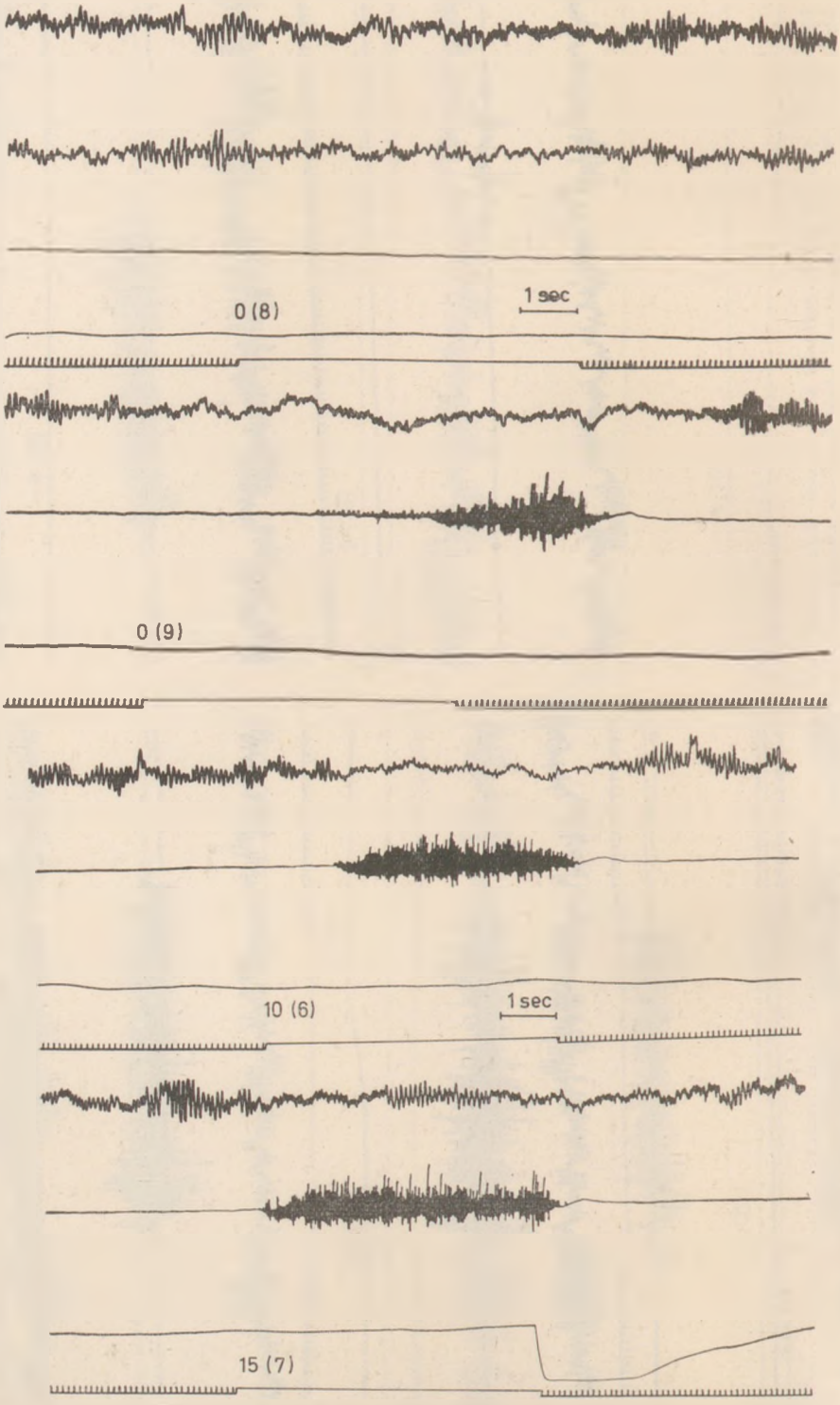

Fig. 14. EEG alpha rhythm blodckae and GSR responses in the zone of 10 db SSP (designation as in *Fig. 12*)

zone — the latency was prolonged sometimes to more than 6 sec *(Fig. 15)*. The GSR amplitude reached two maxima: one with strong sounds, and the other in the threshold zone *(Fig. 16)*. A similar curve was characteristic of the behaviour of alpha rhythm depression.

At given operating levels the latent period did not depend on the given SSP but behaved according to the power law *(Fig. 15)*. At the same time the amplitude of GSR increased on stimuli similar in intensity to the given SSP.

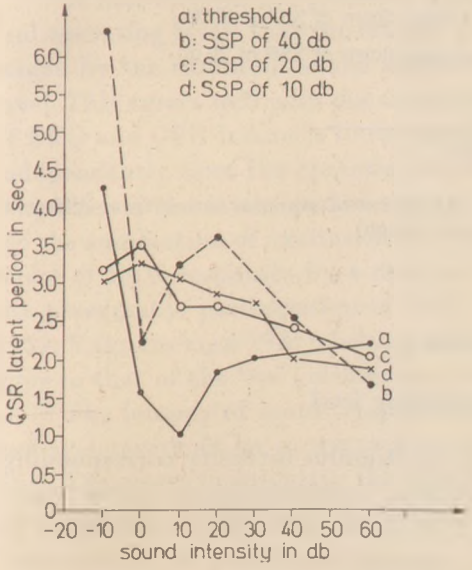

Fig. 15. Dependence on stimulus intensity of GSR latencies at different SSP values (group mean)

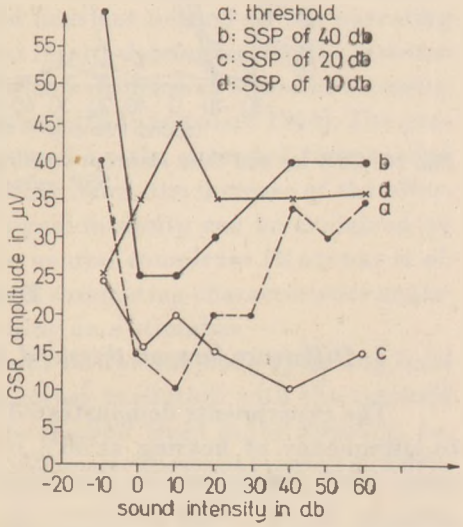

Fig. 16. Dependence on stimulus intensity of GSR amplitudes at different SSPs (group mean)

VI. Dependence on stimulus intensity of hit and false alarm probability of the GSR

GSR responses occurred first of all with strong sounds. At medium intensities they became less frequent, while on weak stimuli their number was unchanged and tended to fall rapidly to zero on approaching intensities below threshold. If no stimulus had been presented, no GSR appeared.

When a SSP of 20 db had been introduced, the curve of hit probability was almost identical with the threshold curve. At SSPs of 40 and 10 db, hit probability reached its maximum in the SSP zone, but it was low at other intensities *(Fig. 17)*. False alarms were infrequent and appeared only at SSPs of 20 and 10 db.

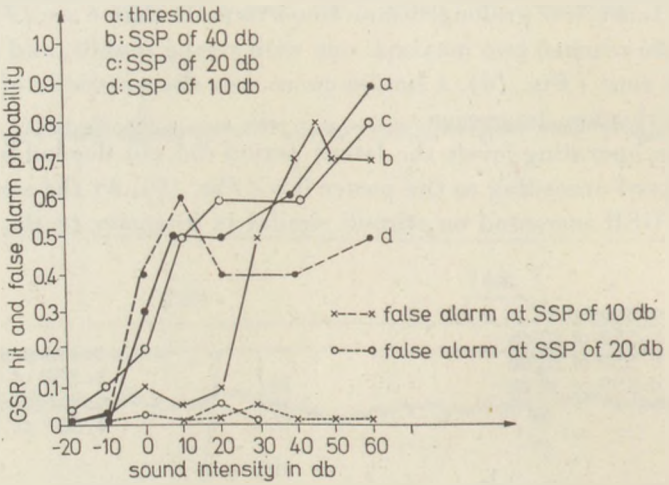

Fig. 17. GSR hit and false alarm probability as a function of stimulus intensity at different SSP values (group mean)

Discussion

I. Difference between threshold an operating level

The experiments demonstrated that the stimulus intensity corresponding to a frequency of hearing at 50% of the time, is practically the mean operating level determined by the retained SSP. If no such SSP is introduced in advance, the operating level develops in a natural way during the experiment. Thus, the level determined as an "absolute threshold" in classical psychophysical studies would correspond to the mean operating level. The presence of EEG and GSR responses independent of the given SSP suggests that besides the operating level used for the accomplishment of a motor response the analyser must have a constant threshold. The operating level may approach the absolute threshold, and can also be shifted towards the area of stimulus intensities beyond threshold.

II. Successive "entrance" of external stimuli into the threshold and operating level

If the EMG and GSR responses are compared with the EMG reactions, the question arises as to the relationship between the analyser's threshold and the operating levels. Presumably, *(a)* the excitation is transmitted on parallel channels and causes complex registerable responses; or *(b)* the excitation surmounts successively the threshold and the operating level. In support of the

second assumption are the experiments of MICHAILEVSKAIA (1964), who on comparing the hit probability curves of EMG and EEG responses to light stimuli, found a typical deviation of EMG curves from the normal distribution characteristic of the EEG responses, which speaks for the successive arrival of external stimuli to the threshold and the operating level.

III. Factors determining the latent period of EEG, GSR and EMG

It follows from the principle of the successive connection of the threshold and operating levels that the latency of EEG and GSR activation can be determined by the threshold of the analyser and does not depend on the operating level. This agrees well with the experimental results demonstrating the increase of EEG and GSR latencies to run parallel with a decrease in stimulus intensity independently from the operating level (KORZH 1963; SOKOLOV 1964). The prolongation of the latent period can be explained by the extended time needed for the summation of excitations. On the other hand, the increase of the after-effect of EEG responses by a decrease of signal intensity can be explained by the presumable participation of "off" type neuron complexes (KATSUKI *et al.* 1959; VARDAPETIAN 1967), having differential summating characteristics analogous to that of the "on" elements at switching on a stimulus.

The latency of motor responses depends on the subject's operating level and is determined by a comparison of an actual excitation with the retained SSP. The closer in intensity the stimulus to the SSP fixed in the memory, *i.e.* the more time is needed for its recognition, the longer the latent period of EMG.

IV. The differential effect of SSPs on EMG latencies and after-effects

If it is true that the latency of EMG is determined by the given operating level, then its after-effect does not depend on it. When the subject decides whether or not a given "signal + noise" corresponds to the criterion, he compares an actual signal with the retained trace of SSP. When he decides, however, whether or not the effect of a stimulus had stopped, then during this kind of comparison the physiological noise is sharply separated from the presented signal. Thus, the possibility of the perception of "noise alone" depends only on the stimulus intensity.

V. The effect of introducing a SSP on the EEG and GSR characteristics

As pointed out above, the introduced SSP had no effect on the latent period of EEG and GSR. In connection with the phenomenon of extinction, however, a tendency can be observed that latencies turn to be levelled up and the hit probability decreases especially at subthreshold stimuli. At the same time,

an effect of the given SSP on EEG and GSR parameters can also be revealed against the background of extinction. This effect has no continuous connection with the analyser's threshold but is determined by the processes taking place in the instant of the comparison of the signal with the retained SSP. The following facts should be taken into consideration: *(1)* the EEG after-effect increases at intensities close to the SSP; *(2)* the EEG and GSR hit probability curves have their peaks in the SSP zone; and *(3)* the curves representing the GSR amplitude plotted against the stimulus intensity also form a number of peaks corresponding to SSPs. This also supports the assumption that EEG and GSR responses as components of the orienting reflex originate from two sources of excitation. One of these is connected with the physical intensity of a stimulus and the other with the comparison process of the retained SSP and the external stimulus.

VI. Sources of false alarm

For estimating false alarm in the EMG indicator we must contrast it with the responses given to stimuli. In normal cases reactions equal in duration to the stimulus predominated. In addition, there exist abortive responses, when no stimulus has been presented. Their duration is between 2.7 and 3.9 sec, but sometimes they lasted 6.6 sec. They may have the following sources. *(a)* A SSP close to the analyser's threshold results in that the "neural noise" in the CNS reaches the operating level. In this case the appearance of false alarms in the higher intensity zone must have a considerably less probability. *(b)* An increase in the excitation of the retained SSP. This mechanism may cause false alarms at stimuli close in intensity to the SSP.

Our experiments have shown that false alarms may occur at SSP introduced in the zone of strong stimuli, too. They suggest that the source of the false alarm may be not only the analyser's "neural noise" but also the fluctuation in excitability of the operating level. In this respect the 6.6 sec false alarm, registered in some cases, presents a valuable proof.

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THE ROLE OF THE PITUITARY IN THE REGULATION OF THE SERUM GLYCOPROTEIN LEVEL

By

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During the development of the turpentine abscess the level of all the glycoprotein carbohydrate fractions increases. The increase is enhanced by pituitary extract. The carbohydrate fractions of glycoproteins undergo a similar change in the liver. The fact that the glycoprotein reaction persists after hypophysectomy is suggestive of a regulation by a phylogenetically ancient regulatory system, presumably through protein induction. On the other hand, the fact that the reaction is under pituitary influence suggests that in higher organisms the phylogenetically ancient regulatory mechanism is subordinated to hormonal regulation, which is controlled by the pituitary.

During the past decades ample evidence has accumulated showing that the serum glycoprotein level is significantly elevated in the widest variety of pathological conditions such as inflammatory, necrotic and proliferative processes, and dystrophic changes (Winzler 1955; Stary 1959; Eylar 1966). The mechanism of this systemic reaction is still not clear, though more and more data indicate that the elevation of the serum glycoprotein level is a result of an increased glycoprotein synthesis with the principal site in the liver (Werner 1949; Budavári and Pósch 1964; Winzler 1965).

Recently, a number of different glycoproteins with specific biological activity has been isolated from serum, for instance haptoglobin, ceruloplasmin, transferrin, thyroxine-binding globulin, *etc.* (Winzler 1955; Stary 1959). This indicates that an elevated serum glycoprotein level is not a by-product, as it was believed earlier, but a biologically purposeful active defensive reaction, an integral part of systemic defence.

Little is known about the control of the glycoprotein level despite the fact that it would be important both theoretically and clinically to know more about the regulation of this important systemic reaction. The observation by Shetlar *et al.* (1955) that in the rat the serum glycoprotein level decreases after hypophysectomy and increases following treatment with pituitary extract seems to prove the role of the pituitary in the regulation of the glycoprotein level. Kushner *et al.* (1956) and Bernasconi (1957) found raised serum glycoprotein levels following ACTH administration. According to Roselund (1958) the increase of the serum glycoprotein level is a systemic response to nonspecific stress, regulated through the pituitary-adrenal system. It is, however, not universally accepted that the pituitary hormones elevate the glycoprotein level.

Boas *et al.* (1955) found an increase of serum glycoproteins following hypophysectomy.

The aim of the present experiments was to supply further data concerning the role of the pituitary gland in the control of the serum glycoprotein level by studying the influence of pituitary function on the elevation of glycoprotein level in response to turpentine abscess as a model inflammation.

Methods

In the experiments 90 male albino rats of our own stock, divided into 5 groups were used.

The 20 rats in Group I were left untreated to serve as controls. The 15 rats in Group II were subjected to parapharyngeal hypophysectomy by the method of Smith (1930), and were killed 3 weeks later. In Group III, 20 animals were injected 0.5 ml of turpentine into one thigh and the animals were killed 72 hours later. In Group IV of 15 rats hypophysectomy was followed 3 weeks later by the induction of turpentine abscess and 72 hours later the animals were killed. Finally, 20 rats in Group V were treated with twice the human substitution dose, *i.e.* 1 ml/100 g body weight of pituitary extract *(Parke Davis)* diluted to 1:700, 4 times at 3-day intervals. Simultaneously, with the last dose of pituitary extract turpentine was also injected and 72 hours later the animals were killed.

The animals were killed invariably by exsanguination from the femoral artery. Of the carbohydrate fractions of serum glycoproteins protein-bound hexose was determined according to Weimer and Moshin (1952), sialic acid according to Svennerholm (1958), and hexosamine by the method of Boas *et al.* (1955). The total protein content of serum was determined according to Lowry *et al.* (1951). From the protein and carbohydrate values the total protein-bound sugar/protein ratio was calculated. In the liver, the principal site of glycoprotein synthesis, hexosamine and sialic acid were estimated.

The serum proteins and glycoproteins were fractionated by agar gel electrophoresis according to the slide method of Grabar and Williams (1955). Proteins were stained according to Smithies (1955), glycoproteins according to Uriel (1960).

Results

The results are shown in the following Figures.

Fig. 1 shows the carbohydrate fractions of serum glycoproteins and the protein-bound carbohydrate/protein ratio. In addition to hexose, sialic acid and hexosamine, fucose, too, belongs to the total carbohydrate bound to protein. Its normal serum level is 8 to 10 mg per 100 ml, and thus it cannot significantly influence the ratio *(Fig. 1)*.

In the hypophysectomized rats, all the three glycoprotein fractions decreased in quantity. The decrease was significant only in the case of protein-bound hexose. In Group III (turpentine treatment) all the three glycoprotein fractions increased significantly, protein-bound hexose by 33%, sialic acid by 54% and hexosamine by 34%. The differences in the increase indicate that there was also a qualitative change in the composition of glycoproteins. An elevation of the glycoprotein level in response to turpentine was demonstrable also following hypophysectomy (Group IV), though the increase was less marked than in Group III, as hexose and hexosamine increased by about 20%, sialic acid by about 40%. In Group V the increase of the glycoprotein level was much

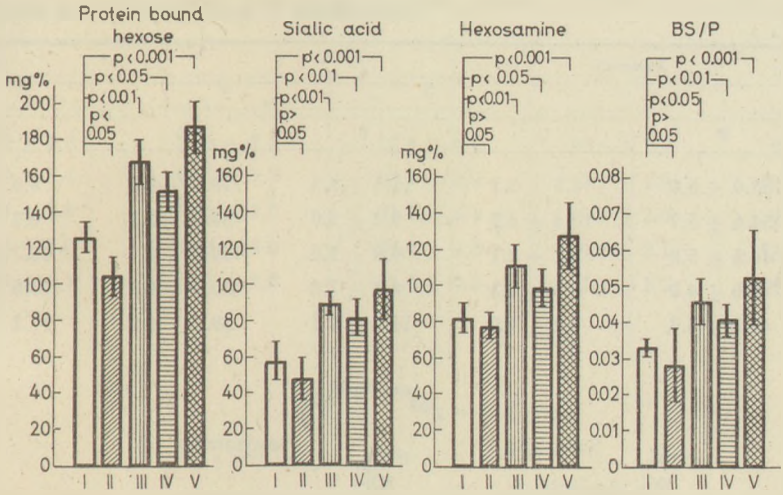

Fig. 1. Carbohydrate fractions of serum glycoproteins and total protein-bound sugar/protein ratio. I: control; II: hypophysectomy; III: turpentine; IV. turpentine + hypophysectomy; V: pituitary extract + turpentine. BS/P = total protein-bound sugar/protein ratio

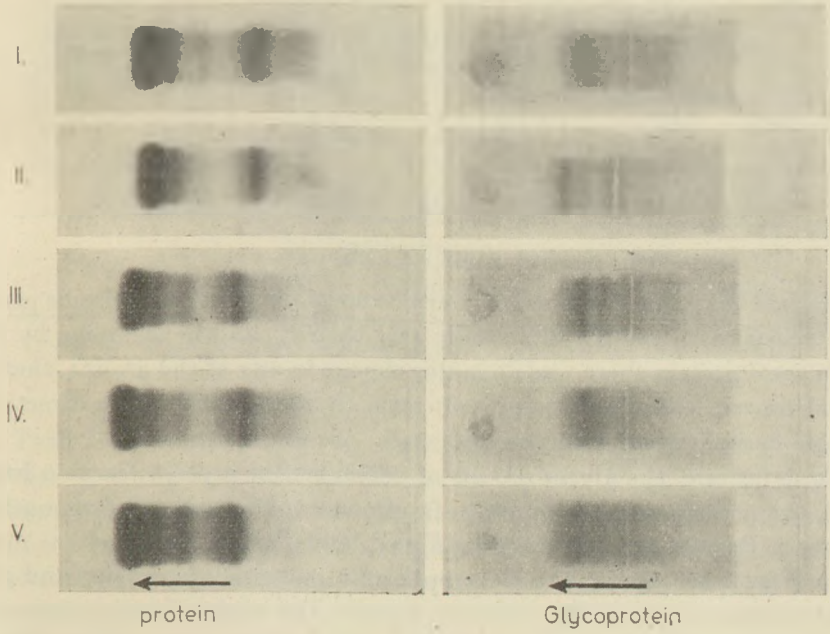

Fig. 2. Agar gel electrophoretic patterns of serum proteins and glycoproteins. (Signs as in *Fig. 1*)

Table I

Distribution of serum proteins and glycoproteins.

	Albumins		Globu-		
			α_1		α_2
	P	G	P	G	P
I	53.0 ± 6.2	8.5 ± 3.1	11.5 ± 2.5	33.3 ± 6.0	8.0 ± 1.8
II	54.6 ± 5.7	10.3 ± 3.8	9.2 ± 2.0	35.3 ± 5.1	6.7 ± 1.6
III	44.5 ± 5.0	4.1 ± 2.1	9.9 ± 2.6	29.0 ± 4.7	15.7 ± 2.0
IV	45.0 ± 4.9	6.3 ± 2.3	8.8 ± 3.0	26.9 ± 4.6	13.6 ± 1.6
V	41.3 ± 5.3	6.1 ± 1.9	11.9 ± 2.1	29.0 ± 4.9	17.2 ± 1.4

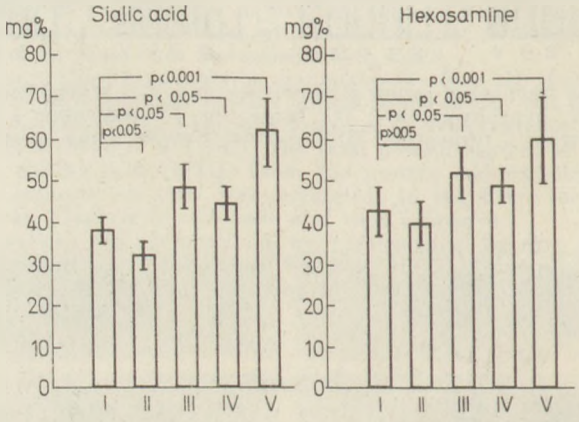

Fig. 3. Sialic acid and hexosamine contents in the liver of rats

more marked than that produced by turpentine alone. In these animals protein-bound hexose increased by 48%, sialic acid by 67% and hexosamine by 55%.

In total protein there was no major change in any of the groups, therefore the total protein carbohydrate/protein ratio changed in the same direction as the single carbohydrate fractions did.

To determine the glycoproteins of which serum protein fraction had increased, the protein and glycoprotein fractions were separated by agar gel electrophoresis. Results are presented in *Figs 2*, and *Table I*.

In *Fig. 2* are shown the electrophoretic patterns of proteins and glycoproteins characteristic of the different groups. The alpha$_2$ fraction was significantly increased in the case of proteins and glycoproteins alike in the rats treated with turpentine (III, IV, V). Hypophysectomy (IV) caused the reaction to weaken while pituitary extract (V) enhanced it.

as determined by agar gel electrophoresis

l i n s

	β		γ	
G	P	G	P	G
20.2 ± 2.9	16.6 ± 2.4	28.8 ± 3.2	10.4 ± 2.2	8.2 ± 1.7
18.5 ± 3.4	17.7 ± 2.9	23.3 ± 3.9	8.2 ± 1.9	12.6 ± 2.3
26.7 ± 3.8	21.6 ± 2.0	31.7 ± 4.1	9.0 ± 2.0	9.0 ± 2.0
24.2 ± 4.0	20.0 ± 3.0	29.0 ± 3.8	9.6 ± 2.1	13.6 ± 2.4
26.9 ± 3.1	22.3 ± 2.6	31.0 ± 3.2	7.3 ± 1.7	6.8 ± 3.0

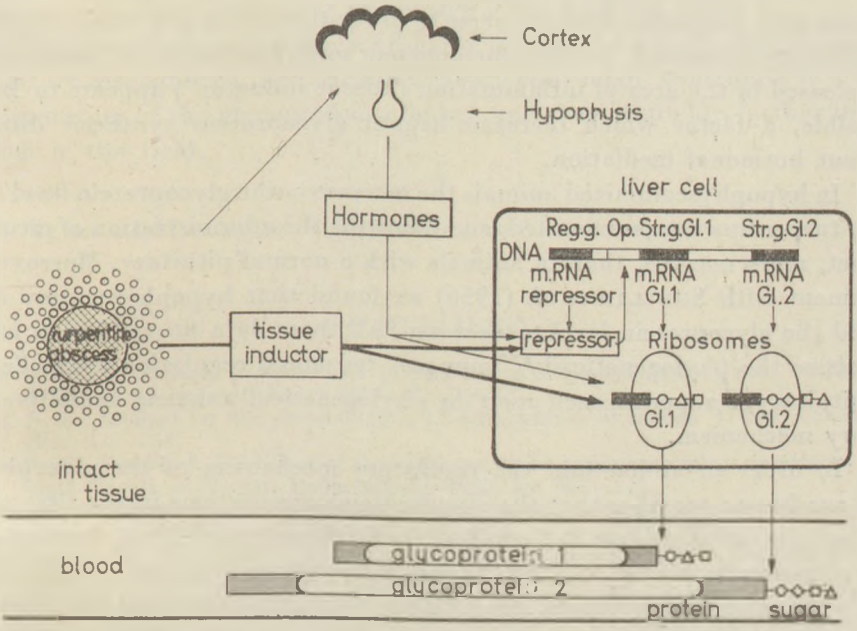

Fig. 4. Hypothetic mechanisms of serum glycoprotein level regulation

Table I shows the percentage distribution of the protein and glycoprotein fractions, with the standard deviations. In the rats treated with turpentine, protein and glycoprotein decreased in the albumin fraction. Protein and glycoprotein increased in alpha$_2$ and beta globulins. The pattern corresponded to that seen in subacute inflammation. In this case too, pituitary extract had a marked effect.

Considering that the main site of glycoprotein synthesis is the liver, its sialic acid and hexosamine contents were estimated, too. Results are shown in *Fig. 3.* In the liver of hypophysectomized animals sialic acid and hexosamine

decreased though the latter change was not significant statistically. Under the effect of turpentine, sialic acid increased by 25% and hexosamine by 21%; both changes were significant. In the animals subjected to hypophysectomy the rise was less marked, while in those treated with pituitary extract the rise was greater than after treatment with turpentine only. These results suggested that pituitary control of the glycoprotein level is effected by an influence on glycoprotein synthesis.

Discussion

The finding of an increased glycoprotein level in hypophysectomized animals with turpentine abscess seems to prove that the change is not mediated by pituitary hormones. For the phenomenon some non-hormonal humoral factor released in the area of inflammation ("tissue inductor") appears to be responsible, a factor which increases hepatic glycoprotein synthesis directly, without hormonal mediation.

In hypophysectomized animals the increase in the glycoprotein level caused by turpentine was less marked, and following the administration of pituitary extract, more marked, than in animals with a normal pituitary. Moreover, in agreement with SHETLAR *et al.* (1956) we found that hypophysectomy alone caused the glycoprotein level to decrease. All these data prove that in higher organisms the phylogenetically "younger" hormonal regulation controlled by the pituitary is superimposed upon the phylogenetically ancient inductive regulatory mechanism.

In order to understand the regulatory mechanism of the glycoprotein level one has to consider that the glycoproteins are proteins in the first place, in which the carbohydrate is a small part of the molecule. Thus, the recent results of molecular biology concerning synthesis and regulation of proteins apply also to the serum glycoprotein. According to WINZLER (1965) and EYLAR (1966) the carbohydrate fraction is a permanent, stable part of the glycoproteid molecule and so from the changes in the carbohydrate content conclusions may be drawn as to those in the molecule as a whole. On the basis of these facts, our hypothesis concerning the regulation of the serum glycoprotein level is as follows *(Fig. 4)*.

As with every protein of the organism, the information on the synthesis of every glycoprotein molecule is carried by a structural gene of the DNA molecule, which through the synthesis of the appropriate mRNA molecule controls the synthesis of glycoproteins in the ribosomes of the liver cells (WINZLER 1965; HALLINAN *et al.* 1967). It is there that the carbohydrate part joins the protein molecule (EYLAR 1966; HALLINAN *et al.* 1967). The above outlined mechanism is supported by the observation that under the effect of

turpentine parallel with the increase of the serum glycoprotein level in the liver the mRNA content and the rate of ^{14}C glycine and ^{14}C-glycosamine incorporation are all increased (CHANDLER and NEUTANS 1964; ROBINSON *et al.* 1964; SARCIONE 1965). These changes do not take place following the administration of actinomycin and puromycin (MAUNG *et al.* 1964; MOLNÁR *et al.* 1964).

According to our hypothesis glycoprotein synthesis and through this a constant serum glycoprotein level would be ensured by the regulatorgen-repressor-operator system described in bacteria by JACOB and MONOD (1961) and in higher organisms by MEERSON (1967). It is either at this point (derepression) or eventually directly at the ribosomal level that the pituitary hormones, and possibly also the hypothetical "tissue inductor", are influencing glycoprotein synthesis. This view seems to be supported by the general mode of action of hormones on protein synthesis (KARLSON 1961; KORNER 1966).

The described mechanism is probably only one of a number of the possible regulatory mechanisms. Yet, the above hypothesis might contribute to a better interpretation of the glycoprotein problem and may stimulate further investigations in this field.

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THE EFFECT OF DEHYDRATION AND REPEATED WATER LOADING ON THE SUPRAOPTIC-NEUROHYPOPHYSEO-NEUROSECRETORY SYSTEM AND THE ADH CONTENT OF THE NEUROHYPOPHYSIS IN THE RAT*

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The effect of water deprivation and of repeated water loading on the antidiuretic hormone activity of the neurohypophysis and the neurosecretory material content of the supraoptic nucleus and the posterior lobe has been studied in the rat.

In normal rats repeated water loading resulted in an increase of ADH activity and in changes of neurosecretory material content of the supraoptic nucleus, characteristic of hypofunction. The opposite was the case in rats deprived of water for 10 days. An increase in number of the "small dark cells" in water deprived rats supported the idea of a difference in the function of these cells and of the larger ones. The results suggested that water loading suppressed posterior pituitary ADH secretion.

Earlier studies indicated that under chronic conditions noxious stimuli resulted in an increase of the hormonal activity of the pituitary posterior lobe (Fendler and Telegdy 1964; Fendler *et al.* 1964, 1966). It was also assumed that the changes in hormonal activity due to different stimuli might be a result of the non-specific activation of the neurosecretory system and the action of adrenocortical steroids released under stressful conditions (Moses 1963, 1964).

It is common knowledge that alterations in the osmolarity of the extracellular space are specific stimuli to the neurosecretory system (Verney 1946; Kovács *et al.* 1951, 1953). There have been observations which suggested a diminution of the neurosecretory material (NSM) and antidiuretic hormonal activity (ADH) in the hypothalamo-neurohypophysial system in response to thirsting and these changes were accompanied by an increase of the ADH content in peripheral blood (see the review of Klement and Cutler 1963). In contrast to this the effect of chronic hydration on the hypothalamo-neurohypophyseo-neurosecretory system cannot be interpreted unequivocally. Andersson and Jewell (1957) reported NSM depletion of the magnocellular nuclei due to hydration associated with an increase in the NSM content along the axons but no remarkable changes in the posterior pituitary. The failure to change of the NSM content of the posterior pituitary can be explained by the fact that it is usually "fully loaded" with Gomori positive material.

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Recent observations on the functional characteristics of the hypothalamo-neurohypophysial system are suggestive of a dissociation of hormonal activity from NSM content in the magnocellular nuclei and the posterior pituitary under certain conditions (Bachrach 1957; Rinné 1960; Moses *et al.* 1963).

The aim of the present investigations was to study the effects of water deprivation and that of repeated hydration on the ADH activity of the posterior pituitary and on the NSM content of the neurosecretory system in the rat.

Methods

The experiments were carried out on a total of 130 Wistar rats of both sexes, weighing 150 to 180 g. They had been fed with purina chow (Gyógyáruért, Budapest) and acclimatized to the laboratory conditions for two weeks prior to the investigations. The experimental procedures and the schedule of investigations were as follows.

1) After being subjected to total water deprivation with food given ad libitum for a 10-day period, 15 rats were sacrificed for the estimation of the ADH activity of the posterior pituitary and for histological study of the NSM content of the neurosecretory system. The posterior pituitary of 3 rats was also studied histologically.

2) A group of 46 rats was subjected to water deprivation for 10 days; for hydration, tap water in an amount of 10% of the body weight was given through a gastric tube daily for five consecutive days, and urine flow was measured in 30 min fractions for 5 hours. The animals were killed on the 1st, 2nd, 3rd and 5th day of hydration for the estimation of posterior pituitary ADH activity and for histological study of the neurosecretory system. The posterior pituitary of 3 rats from each daily group was studied for NSM content.

3) Fifty normal rats were subjected to water loading according to the same schedule as the second group.

4) Nineteen normal rats (without water loading or deprivation) were used as controls.

After extraction with 0.25% acetic acid, the estimation of posterior pituitary ADH activity was performed according to the method of Dekansky (1952). For the demonstration of the NSM content, sections of both control and experimental specimens were placed on the same slide and stained with Gomori's aldehyde-fuchsin (1950) as modified by Gabe (1953). Urine flow was measured with 0.1 ml, adrenal weight with 0.1 mg, and posterior pituitary weight with 0.01 mg accuracy. Student's *t* test was used for evaluation.

Results

Fig. 1 shows urine flow during the 5-day hydration period. The diuretic response of the normal rats showed no significant alteration during the hydration period. In contrast, there was no diuretic response due to water loading in water deprived rats during the first 2 days of hydration. Then a gradual increase in water excretion followed but water retention still amounted to more than 60% on the 5th day.

Table I demonstrates posterior pituitary ADH activity and adrenal weight in the course of the experiment. Taking into consideration that in the animals either deprived of or loaded with water body weight changed significantly, ADH activity and endocrine organ weights were expressed not only on the basis of 100 g body weight but also in absolute values. Statistical evaluation and comparison of the data in respect of ADH activity were made on the basis of mU per organ.

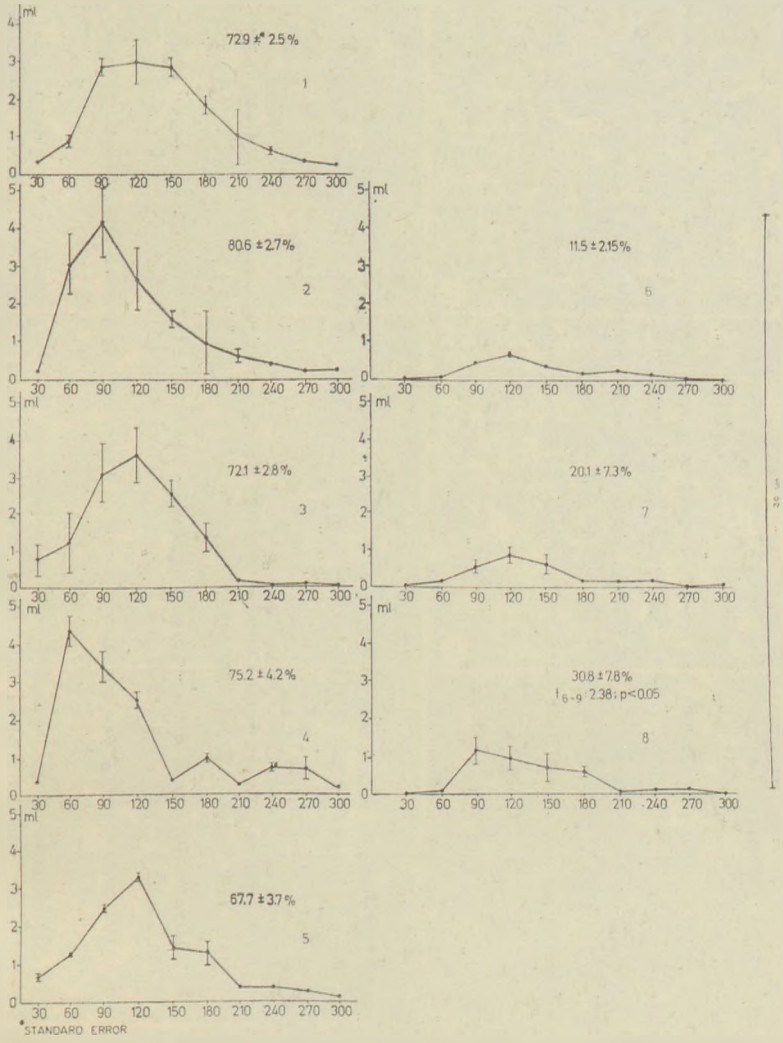

Fig. 1. Result of daily water loading. Urine flow of normal animal: 1) first; 2) second; 3) third; 4) fourth; 5) fifth water loading. Urine flow of water-deprived animal: 6) third; 7) fourth; 8) fifth water loading. The figures above the diagrams indicate the volume of urine in per cent of the water load during the 5-hour observation period

Hydration caused posterior pituitary ADH activity to change in normal rats after the first two water loadings, leading to a marked increase in the next 3 days. In contrast, ADH activity decreased in the rats deprived of water for 10 days, and water loading induced a variable but definite increase of ADH until the 5th day of hydration. Posterior pituitary weight increased considerably during water deprivation but could be restored by repeated water loadings in a few days. Similarly, adrenal weight in the water deprived rats was higher than

Table I

No. of animals	Body weight, g	ADH/NL	ADH/100 g bw.	ADH/1 mg NL w.	ADH/100 g bw/1 mg NL w.	NL w.	NL w/100 g bw.	ADR w.	ADR w/100 g bw.
			1. ADH content, neural lobe and adrenal weight of normal control animals						
16	160 ± 4	579 ± 44	364 ± 20	480 ± 36	360 ± 16	1.18 ± 0.05	0.75 ± 0.04	33.8 ± 1.38	21.4 ± 1.64
			2. ADH content, neural lobe and adrenal weight of normal animals after one water load						
10	156 ± 11.5	558 ± 84	370 ± 31	479 ± 53	314 ± 22.2	1.14 ± 0.08	0.74 ± 0.02	33.3 ± 1.81	22.3 ± 0.80
			3. ADH content, neural lobe and adrenal weight of normal animals after two water loads						
10	157 ± 14	555 ± 110	420 ± 71	450 ± 52	374 ± 73	1.34 ± 0.02	0.86 ± 0.01	34.7 ± 19	22.8 ± 1.7
			4. ADH content, neural lobe and adrenal weight of normal animals after three water loads						
6	179 ± 15.7	1241 ± 155 t_{1-4}: 4.11; $p < 0.001$	696 ± 108	806 ± 109	469 ± 95	1.5 ± 0.1	0.86 ± 0.02	60 ± 10	33.5 ± 4.2 t_{1-4}: 2.69; $p < 0.02$
			5. ADH content, neural lobe and adrenal weight of normal animals after five water loads						
12	163 ± 7.4	1262 ± 129	769 ± 27.5	1128 ± 157	737 ± 131	1.14 ± 0.08	0.68 ± 0.008	39 ± 2.85	23.7 ± 0.95
			6. ADH content, neural lobe and adrenal weight of animals deprived of water						
12	107 ± 3.5	151 ± 13.7 t_{1-6}: 9.31; $p < 0.001$	149 ± 17.8	108 ± 84	115 ± 11.3	1.47 ± 0.06 t_{1-6}: 3.62; $p < 0.01$	1.37 ± 0.03	44.4 ± 3.0 t_{1-6}: 3.21; $p < 0.01$	41.8 ± 3.2 t_{1-6}: 5.66; $p < 0.001$
			7. ADH content, neural lobe and adrenal weight of water deprived animals after one water loads						
6	123 ± 10.9	150 ± 26.4	116 ± 11.8	104 ± 20	80 ± 9.2	1.46 ± 0.07	1.2 ± 0.14	43.6 ± 7.5	36.5 ± 5.1
			8. ADH content, neural lobe and adrenal weight of water deprived animals after two water loads						
7	129 ± 6.7	204 ± 21.4	154 ± 11.7	139 ± 12.8	107 ± 8.3	1.41 ± 0.04	1.1 ± 0.04	40.6 ± 3.5	31.7 ± 3.8
			9. ADH content, neural lobe and adrenal weight of water deprived animals after three water load						
14	112 ± 5.7	126 ± 13	122 ± 19	89 ± 20.3	95 ± 16.5	1.37 ± 0.03	1.3 ± 0.02	38.5 ± 1.58	36.4 ± 2.87
			10. ADH content, neural lobe and adrenal weight of water deprived animals after five water loads						
7	119 ± 9.5	252 ± 22.5 t_{6-10}: 3.88; $p < 0.01$	234 ± 28	275 ± 38.8	260 ± 44.7	0.92 ± 0.02	0.82 ± 0.02	36.5 ± 2.21	31 ± 1.0

Mean ± standard error of the mean

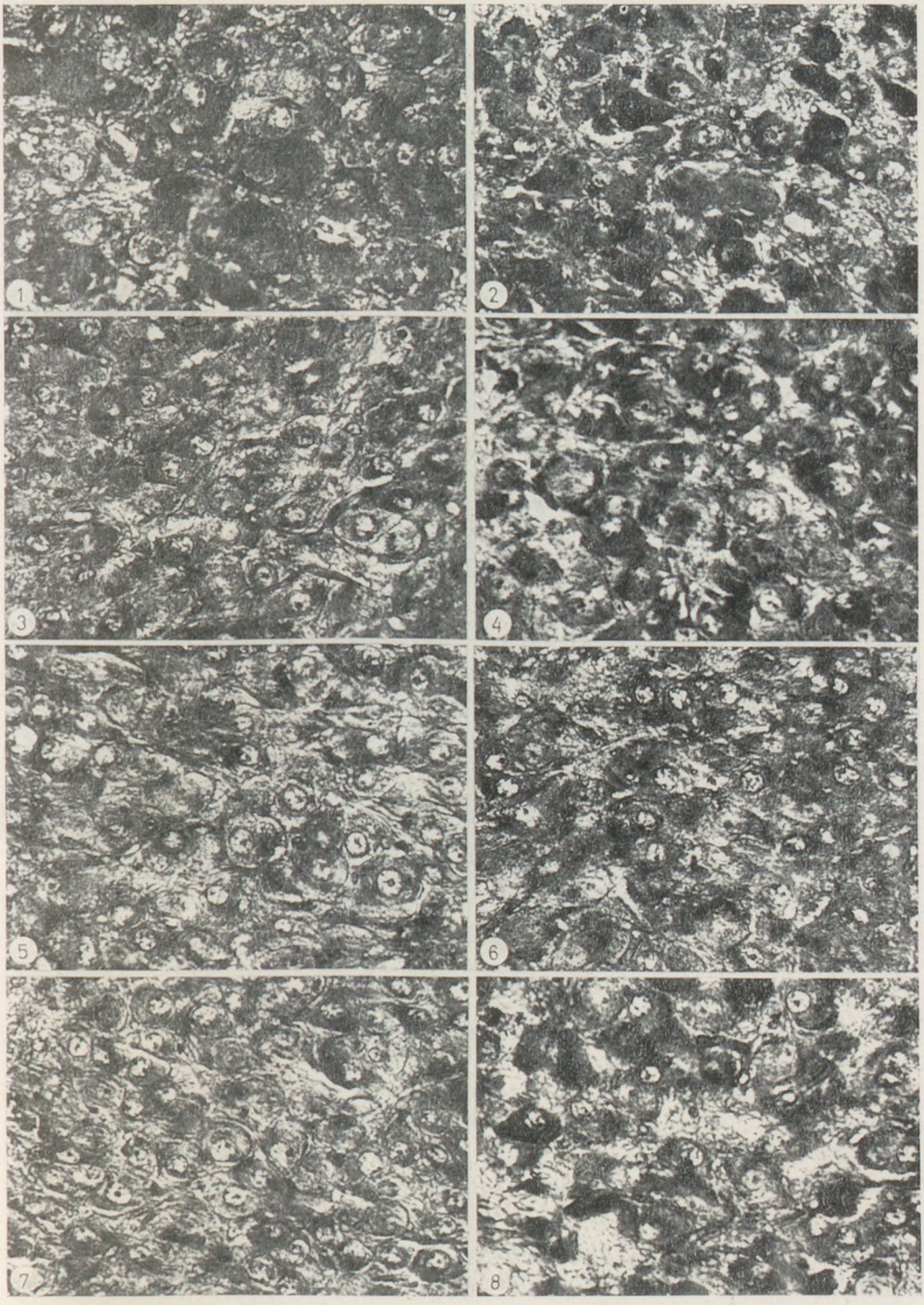

Fig. 2. Supraoptic nucleus. 10 μ thick sagittal sections. Aldehyde-fuchsin staining. ×315. 1) Normal animal. 2) water-deprived animal. 3) after one water load. 4) after water deprivation followed by one water load. 5) after two water loads. 6) after water-deprivat on followed by two water loads. 7) after three water loads. 8) after water deprivation followed by three water loads

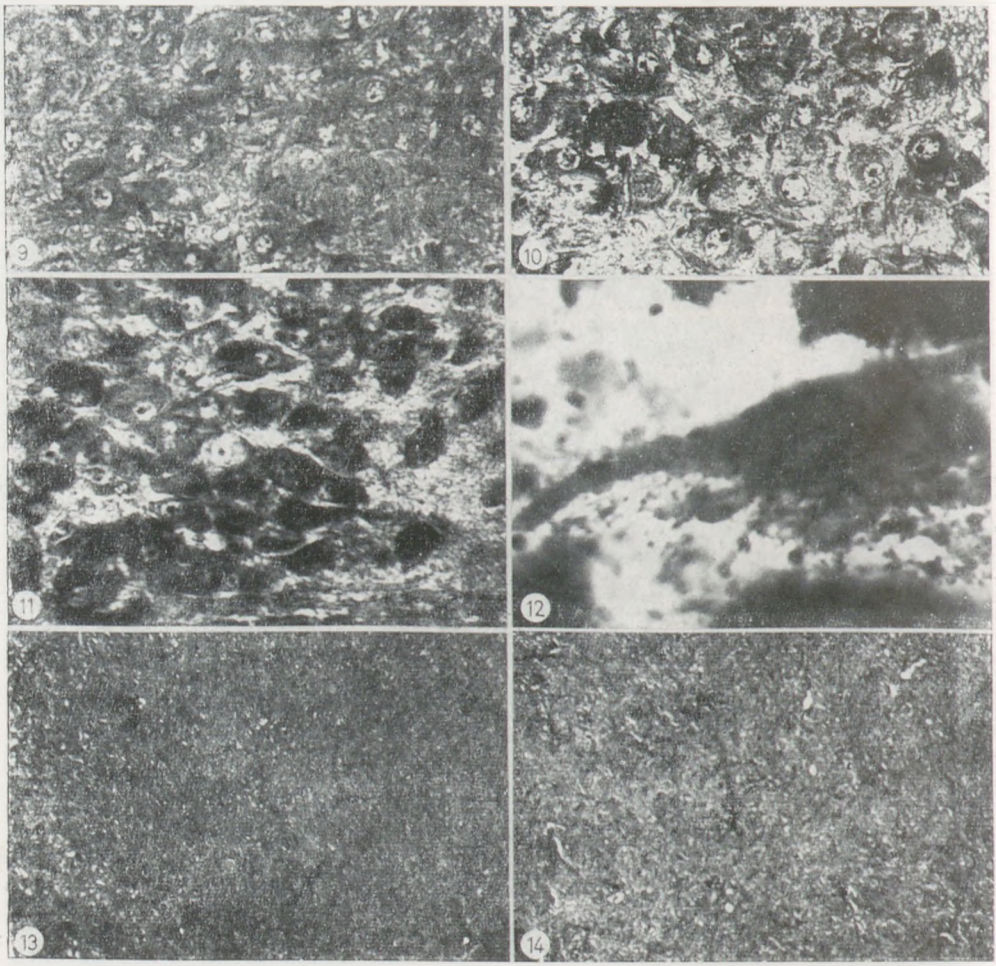

Fig. 3. Supraoptic nucleus, 10 μ thick sagittal sections. Aldehyde-fuchsin staining, $\times 315$. 9) After five water loads. 10) after water deprivation followed by five water loads, 11) water deprived animal. "small dark cells". 12) water-deprived animal. "small dark cell". $\times 1575$, 13) Posterior pituitary of normal animal, 5 μ thick sagittal section, $\times 100$. 14) Posterior pituitary of water deprived animal, 5 μ thick sagittal section. aldehyde-fuchsin staining, $\times 100$

in the controls, and water loading led to a diminution of adrenal hypertrophy. Some normal rats displayed enlarged adrenals after hydration, which indicated that the excess water intake had activated the pituitary-adrenocortical axis.

The NSM content of the neurosecretory system of water-deprived, water-loaded and control rats is shown in *Figs 2* and *3*. Water deprivation led to a hyperactivity of the neurosecretory system, which manifested itself in enlarged nucleoli, in a perykarial distribution of the NSM, and in an increase in the axon-

ally located droplets of Gomori positive material. In contrast, the normal rats subjected to hydration displayed NSM depletion of the neurosecretory system, although posterior pituitary ADH activity was elevated in these experiments.

An interesting finding in the water-deprived rats was the increase in number of the "small dark cells" (STREEFKERK 1967) in the neurosecretory system. These cells, which stained intensely with aldehyde-fuchsin, were characteristic of the supraoptic nucleus of water-deprived animals, and their number was reduced under the effect of water loading.

Discussion

Changes in the NSM content of the magnocellular nuclei of the hypothalamus as well as in hormonal activity in response to osmotic stimuli have been known for long. A decrease in the NSM content due to water deprivation was observed in the posterior pituitary in agreement with earlier data (HILD and ZETLER 1953; LEVEQUE and SCHARRER 1953; STREEFKERK 1967; *etc.*). An enlargement of the nucleoli in the cells of the supraoptic nucleus and an increase of the Gomori positive material in the Golgi area might be interpreted as signs of hypersecretion (ORTMANN 1951; ERDSTRÖM and EICHNER 1958; IFFT and BERKOVITZ 1965; STREEFKERK 1967). NSM depletion of the magnocellular cells in response to water deprivation is a complex process; according to the present observations the NSM shows a perykarial distribution in the cells of the supraoptic nucleus and an accumulation in the area of the axon hillock in thirsted animals. Redistribution of the aldehyde-fuchsin positive material around the nuclei of neurosecretory cells after water loading could not be clearly observed in the present investigations. The failure of the morphological picture to return to normal can be explained by the fact that after the long-continued water deprivation hydration of relatively short duration might be insufficient to normalize the distribution of the NSM.

The "small dark cells" described by STREEFKERK (1967) were significantly increased in number in the supraoptic nucleus of water-deprived rats and, in contrast to the large magnocellular cells, were loaded with NSM. The distinction between the larger but depleted cells and the small dark cells in water-deprived animals raises several questions. Is there any difference in function between the two cell types or are the small dark cells transformed from the larger ones as a result of water deprivation? The inhomogeneity of the cells in respect of size and NSM content and their distinct distribution in the supraoptic nucleus were also observed by ANDERSSON and JEWELL (1957), and the increase in number of the small dark cells along the caudo-cranial axis of the supraoptic nucleus in the course of water deprivation seems to justify a differentiation between the small dark and the large light cells.

Posterior pituitary ADH activity showed a parallelism with the histological findings in the water-deprived animals. In these rats, as a result of the increased release of the hormone into blood, posterior pituitary ADH activity was considerably reduced and the morphological picture of the neurosecretory system was indicative of hypersecretion. In water-loaded normal rats the inhibition of ADH release was accompanied by a decreased secretory function of the supraoptic nucleus as shown by its Gomori positivity. Involvement of the adrenocortical hormones in the inhibition of ADH release under the effect of water loading has been described (Moses 1963, 1964); an excessive increase in extracellular space leads to adrenal enlargement, and adrenocortical hypersecretion might also be implicated in the inhibition of ADH release. Hypervolaemia acting through vascular receptors is also a factor which participates in the control of ADH secretion (Share 1967). Predominance of any one of these multiple regulatory mechanisms in ADH release during water loading is a subject of speculation today and requires further investigations.

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THE RENAL EFFECTS OF HYPERTONIC MANNITOL IN WATER DEPRIVATION

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Experimental dehydration was induced in dogs deprived of water but allowed free intake of food, by giving 20% mannitol, 10 ml/kg b.w., in daily intravenous injections.

It has been established that *(i)* hypertonic mannitol aggravates dehydration; *(ii)* despite severe dehydration, the fractional rejection of water and sodium increases progressively; *(iii)* the infusion of mannitol abolishes the tubular compensatory reactions characteristic of water deprivation; *(iv)* gross reduction of GFR occurs more rapidly than in dehydration not accelerated by mannitol.

The obtained results support the concept suggested previously that the renal response to water deprivation consists of two well-defined components, *i.e.* a tubular and a glomerular one; the former can be abolished by repeated infusions of mannitol. and this will cause the manifestations of dehydration to ensue much more rapidly.

In a previous work from this laboratory (Forgács *et al.* 1968) the renal response to water deprivation was studied in dogs allowed free access to food. It has been established that *(i)* the kidney retains its maximum concentrating ability even after 8 to 12 days of water deprivation; *(ii)* GFR is not reduced before the 4th day of water deprivation, when the volume of extracellular fluid has already decreased to 80% of the control value; *(iii)* the fraction of filtered water and sodium rejected by the tubules decreases progressively in the course of dehydration; *(iv)* the tubular reabsorption of sodium is increased more than that of water. From these results it has been concluded that, in the initial phase of dehydration, the compensatory activity of the kidney involves mainly tubular factors and is characterized by maximal concentration, whereas in the second phase the diminution of GFR reduces the excreted amount of water and sodium, which is in favour of delaying the lethal consequences of water deprivation.

In an attempt to clarify the above mechanism of the dehydration reaction, experiments were performed in which the tubular component of the renal compensatory effort was prevented by combining water deprivation with the administration of hypertonic mannitol.

Mannitol is known to augment the renal excretion of water and sodium. Increased salt loss is an indirect consequence of water trapping by mannitol in

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the renal tubules, which reduces the concentration of sodium in the tubular fluid and hence increases the transtubular gradient, thereby limiting active reabsorption, and enhancing the passive flux towards the lumen, of sodium. The augmented excretion of water is partly due to the osmotic trapping of water in the lumen, and partly to the washout of the medullary osmotic gradient (Wesson and Anslow 1948; Rapoport *et al.* 1949; Malvin and Wilde 1959; Goldberg *et al.* 1964).

Methods

Experiments were performed on 7 female mongrel dogs weighing 12 to 16 kg; prior to the study, perineotomy was carried out which allowed catheterization of the bladder. The animals were kept in metabolism cages. On three subsequent days, control measurements were made, during which free access to water and food was allowed. Water was then withdrawn, and the usual diet was replaced by dry stewed beef. Water deprivation was continued for 4 days, during which 20% mannitol was given in intravenous injections of 10 ml/kg b.w. daily. Urine was collected in 24-hr periods. The concentration of creatinine, sodium and potassium in plasma and urine, plasma and urine osmolalities, serum NPN and the haematocrit were estimated daily. In 5 dogs, inulin space was determined in the control period and on the 3rd day of water deprivation and mannitol treatment, using the one-injection technique described by Bálint *et al.* (1947).

Four days after water had been withheld and mannitol treatment started, the dogs were anaesthetized with sodium pentobarbital (30 mg/kg intravenously); from an abdominal midline incision, the left renal pedicle was approached, and the renal vein was connected with the external jugular vein by a siliconized plastic tube. A T-extension of the tube allowed direct estimation of renal blood flow (RBF_{air}). Further methodical details have been described in a previous paper (Forgács *et al.* 1968).

All parameters of renal function were computed for 100 g of kidney, except for renal vascular resistance (R_{ren}) which was computed for 1 kg of kidney. Statistical analysis of the results was undertaken by using Student's *t*-test.

Results

Table I summarizes the results (mean + S.E.M.) obtained in the control period and during water deprivation combined with infusion of 20% mannitol daily.

Urine flow was twice the control value on the 2nd day of water deprivation, showing a subsequent diminution towards the end of the experiment.

Glomerular filtration rate (GFR), after a slight and statistically not significant increase, decreased to very low values, attaining 26% of the control mean on the 4th day of water deprivation ($P < 0.01$).

The interference of mannitol with tubular function was clearly shown by the progressive diminution U_{creat}/P_{creat}. The urine to plasma osmolal ratio (U_{osm}/P_{osm}) increased somewhat on the 2nd day of water deprivation and mannitol treatment, but the osmotic peak had not been reached on the 4th day of the experiment. The fraction of filtered sodium and water rejected by the tubules (TRF_{Na} and TRF_{H_2O}) exhibited a progressive increase; urinary sodium excretion ($U_{Na}V$) increased initially, but this was followed by a reduction.

Table I

The effect of water deprivation combined with the administration of 20% mannitol in daily intravenous injections of 10 ml/kg b.w., on various parameters of renal function, haematocrit, non-protein nitrogen, and extracellular fluid volume

n = 7	Control		2nd day		3rd day		4th day	
	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.
V ml/min	0.52	0.10	1.07	0.26	0.45	0.05	0.28	0.05
C_{creat} ml/min	39	7	50	19	22	6	10	3
U_{creat}/P_{creat}	78	11	63	11	47	12	37	13
P_{Na} mEq/l	145	2	149	3	152	2	179	4
P_K mEq/l	4.8	0.2	5.0	0.2	5.1	0.2	4.8	0.2
$U_{Na}V$ mEq/l	112	45	182	65	58	14	23	5
TRF_{Na} per cent	1.92	0.55	1.71	0.45	2.09	0.77	3.31	1.25
TRF_{H_2O} per cent	1.67	0.32	1.69	0.25	2.77	0.53	4.25	1.57
U_{osm}/P_{osm}	2.58	0.20	3.12	0.51	3.03	0.37	2.99	0.43
Ht per cent	48	2	49	2	53	3	57	5
NPN mg per 100 ml	33	2	52	3	53	4	72	7
*EC volume ml/kg	238	15			194	21		

*n=5

Table II

Comparison of the values for blood pressure, renal blood flow, renal vascular resistance, and creatinine clearance in a series of normal dogs and in dogs subjected to water deprivation and mannitol treatment for 4 days (data derived from BÁLINT and FORGÁCS 1967)

	Control			Water deprivation + mannitol treatment		
	Mean	S.E.M.	n	Mean	S.E.M.	n
BP mmHg	128	3	78	95	5	24
R_{ren}	1.99	0.09	78	2.60	0.17	24
RBF ml/min	421	14	78	237	18	24
C_{creat} ml/min	52	3	71	14	2	24

Elevation of plasma sodium concentration indicated the ensuing hyperosmotic reduction of body fluids; on the 4th day of water deprivation, the mean attained 180 mEq/l. The progressive increase of the haematocrit from an average of 47% to 57% also showed the decrease of plasma volume. Inulin

space measured on the 3rd day of dehydration decreased to 80% of the control value. Serum NPN level showed a slight increase.

Values for RBF and GFR revealed by direct estimation on the 4th day of the experiment were compared to data obtained in a series of normal dogs, using the same techniques of determination (BÁLINT and FORGÁCS 1967). As shown in *Table II*, water deprivation and the administration of mannitol reduced RBF_{dir} and GFR_{dir}. Renal vascular resistance increased substantially. The differences for all three parameters were significant statistically ($P < < 0.001$).

Discussion

The present results indicate that the administration of large amounts of hypertonic mannitol to dogs deprived of water considerably alters the dehydration reaction, accelerating the whole course of pathologic events. The renal response to water deprivation and mannitol loading follows partly the pattern of the dehydration reaction (KERPEL-FRONIUS 1935; ELKINTON and DANOWSKY 1955), and is partly a consequence of the administration of large amounts of an osmotic diuretic.

The reaction to water deprivation is characterized by the elaboration of urine small in volume and high in osmolality, reduction of sodium excretion, hypernatraemia, and haemoconcentration. The renal response is directed to the maintenance of body fluid volume, *i.e.* all efforts are made to delay the decrease in extracellular fluid volume (KERPEL-FRONIUS 1957).

The administration of mannitol causes extracellular fluid expansion, dilution of plasma, and increased renal excretion of sodium; these changes lead to hyponatraemia within a few hours (TARAIL *et al.* 1951; MOORE 1963; GALAMBOS *et al.* 1963). Long-term administration of mannitol is associated with a high urinary water/electrolyte ratio. A considerable fraction of urine osmolality is shared by the non-resorbable solute, therefore, the excretion of strong electrolytes is depressed which, even in face of increased water output, leads to hypernatraemia (GIPSTEIN and BOYLE 1965).

In the present experiments, urine flow increased on the 2nd day of water deprivation and mannitol treatment, obviously under the effect of the osmotic diuretic. Subsequently, urine output diminished. This, together with the depressed GFR and elevated TRF_{H_2O}, indicated that trapping of water by mannitol in the renal tubules occurs even in severe dehydration. Urine flow, however, was reduced owing to the diminution of GFR.

The renal excretion of sodium was, after an initial rise, significantly depressed, whereas TRF_{Na} increased. This again shows that sodium is trapped under such conditions in the tubules, but an appropriate reduction of the filtered load results in a diminution of netto sodium output.

The initial rise of GFR may have been due to the expansion of plasma volume by the mannitol. The subsequent considerable drop in GFR was due to the haemoconcentration and deterioration of systemic circulation and, probably, to an increased activity of the renin-angiotensin system. This was supported by the nearly 50% diminution in RBF and by the substantial increase in renal vascular resistance.

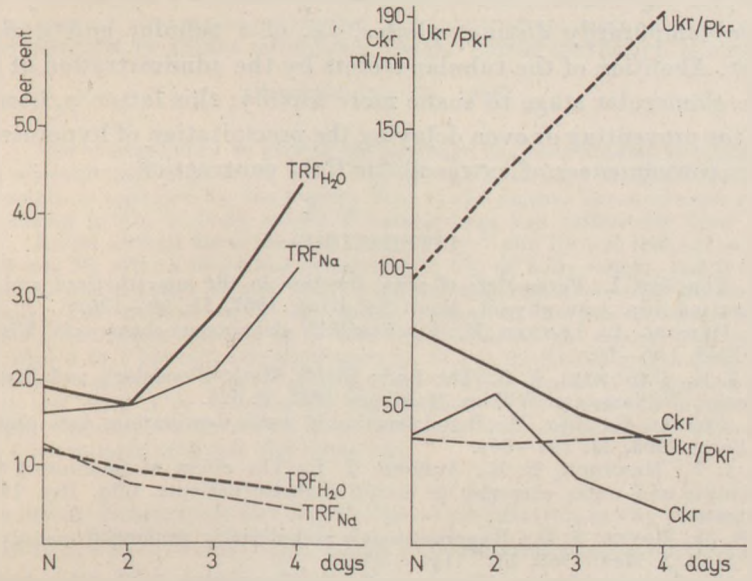

Fig. 1. Comparison of the effects of water deprivation with and without the administration of mannitol. Solid lines represent the findings obtained in water restriction combined with mannitol infusion, broken lines show the results obtained in a previous series of experiments (FORGÁCS *et al.* 1968) where mannitol was not given. *Abscissa*, time in days; *left ordinate and diagram*, changes in sodium and water excretion, in per cent of filtered loads; *right ordinate and diagram*, alterations in creatinine clearance and U_{creat}/P_{creat}

Figure 1 summarizes the results obtained in a previous experimental series (FORGÁCS *et al.* 1968) where water deprivation was not combined with the repeated infusion of mannitol, and the results of the present study. While simple water restriction is accompanied by a continuous rise of U_{creat}/P_{creat} which indicates that the kidney concentrates urine to a maximal degree, the infusion of mannitol during water deprivation caused this ratio to diminish. The impairment of renal concentrating ability was reflected also by the opposite alterations of TRF_{Na} and TRF_{H_2O} in the two experimental series: whereas the fraction of filtered sodium and water rejected by the tubules decreased in water deprivation, a very marked increase of these parameters was observed when mannitol had also been given. When water was restricted but no mannitol was given, GFR remained essentially unchanged during the first 4 days of water deprivation, whereas considerable diminution occurred on the 3rd

day when the dehydration reaction had been enhanced by the administration of mannitol. These findings indicate that the infusion of mannitol during water deprivation abolishes the tubular factors of the renal compensatory mechanism, consequently, the dehydration reaction is precipitated and the only means by which the kidney is capable of reducing fluid loss is a reduction of GFR. The observations support our hypothesis advanced previously that, in water deprivation, the compensatory efforts of the kidney are made up of two temporarily distinct phases, *i.e.* of a tubular and a glomerular component. Abolition of the tubular factors by the administration of mannitol causes the glomerular stage to ensue more rapidly; this latter is, however, insufficient for preventing or even delaying the precipitation of hyperosmosis and the serious consequences of extracellular fluid contraction.

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SURVIVAL OF PIGEON AFTER GRADED HAEMORRHAGE

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The susceptibility to graded haemorrhage has been studied in unanaesthetized and anaesthetized pigeons. A blood loss up to 5% of body weight, which is fatal in mammals, is tolerated by the pigeon. Most of the pigeons survived even a blood loss amounting to 8% of body weight if haemorrhage had sufficiently been protracted.

Initial arterial blood pressure being 101 ± 7 mm Hg was reduced to an average of 68 mm Hg after a blood loss accounting to 5% of body weight, and it returned to about 80 mm Hg after 30 min. Restitution was apparent even after the second haemorrhage. The normal value for cardiac output in the pigeon attained 78.2 ± 59 ml/100 g/min. After bleeding up to 5 or 6% of body weight cardiac output decreased, and after 3 to 4 hours it increased again to 41.1 ± 33 ml/100 g/min. Total peripheral resistance was 11.6 ± 0.9×10^3 dyne sec · cm^{-5} which increased in response to haemorrhage and after 3 to 4 hours was still elevated (12.8 ± 0.9×10^3 dyne sec · cm^{-5}). Oxygen consumption decreased from the initial 18.4 ml/kg/min to 13.4 ml/kg/min, and 4 hours later attained 15.9 ml/kg/min.

In a study concerning the regulation of circulation in the pigeon hypotension was induced by haemorrhage. These experiments suggested that blood loss of a degree which is invariably fatal in mammals causes only transient circulatory changes in the pigeon.

The consequences of haemorrhage on mortality have not been studied in birds and in the literature no data about standardized haemorrhagic shock could be found. Schermer (1954) studied the effect of experimental haemorrhage on blood counts and on the production of formed elements, and concluded that restitution was more rapid in birds than in the rat.

The maximal bleeding volume has been estimated by numerous investigators (Davis and Coe 1954; Kotula *et al.* 1957, *etc.*). Bleeding volume is affected by a variety of factors such as acute exposure to cold or heat, changes in thyroid function, adrenaline (Newell and Shaffner 1950a), *etc.*, and is increased by starvation (Baker *et al.* 1950; Barlow and Whitehead 1928/29).

Bond (1958) succeeded in showing that the circulating blood volume was somewhat greater in birds than in mammals. For the pigeon he found an average blood volume of 9.2 ± 0.3 ml/100 g by measuring plasma volume with the dye *T 1824* and parallelly estimating the haematocrit value. Remarkably, blood volume of the domestic fowl and the pheasant was smaller whereas that of the diving birds larger than the blood volume of mammals; from this it was concluded that adaptation to flying or diving increases blood volume (Bond 1958).

ANDERSEN (1966) reported the blood volume of the pigeon to be 7.0 ml/100 g. Blood volume is subject to variations according to age (MEDWAY and MARLEY 1959; NEWELL and SCHAFFNER 1950a; STURKIE *et al.* 1953), sex (BARLOW and WHITEHEAD 1928/29; KOTULA *et al.* 1957; NEWELL and SCHAFFNER 1950b), the amount of adipose tissue (NEWELL and SCHAFFNER 1950a), and body weight (KOTULA and HELBACKA 1966a, b).

In the present study the survival rate, blood pressure and cardiac output have been estimated both in unanaesthetized and anaesthetized pigeons after the withdrawal of known amounts of blood.

Methods

Experiments were undertaken on 178 pigeons of either sex and of different strains, breed and age, weighing 230 to 500 g. The birds were deprived of food 22 to 24 hours prior to the experiment but free access to water was allowed.

The animals were arranged in two groups.

I. Bleeding of the unanaesthetized pigeon. Without anaesthesia, varying amounts of blood (5 to 8 ml/100 g of body weight) were drawn by amputating a toe. Bleeding was undertaken in two phases. The duration of haemorrhage depended on the intensity of bleeding and usually attained 10 min. An interval of at least 1 hour was instituted between the two bleedings. After bleeding a tight dressing was placed on the injured toe. The animals received no anaesthetic or sedative, and no heparin. In addition to survival rate and body temperature, the effects of water deprivation and muscular exertion (forced flying) were also studied.

II. Bleeding of the anaesthetized pigeon. In this group anaesthesia was induced by intramuscular injection of 1 to 1.5 g/kg urethane.

The feathers were removed from the neck and the ventral surface of the wing, making thereby the basilic vein visible and accessible to injections. An incision was made on the skin by an electrocauter and the right carotid artery was exposed on the neck. Heparin was injected into the wing vein to inhibit coagulation of blood. Blood pressure was measured by a mercury manometer through a polyethylene cannula inserted into the carotid artery. Blood was removed through a cannula from the carotid artery. Bleeding was performed in two phases, with an interval of 30 min between the two bleedings which lasted 10 min each. The amount of blood thus removed attained 5 to 6 ml/100 g of body weight. In some of the animals cardiac output, and in a few cases oxygen consumption was also measured.

Cardiac output was estimated in 36 pigeons by the dye-dilution technique of STEWART and HAMILTON, using T-1824 (BULLARD 1959).

Oxygen consumption was measured in 8 pigeons by a *Noyons-type* diapherometer (Kipp, Delft, Holland) and recorded on a Kipp Micrograph.

Statistical analysis of the results was undertaken using STUDENT's *t*-test.

Results

Table I shows the results obtained in the group not subjected to anaesthesia during bleeding. Remarkably, in this group after the removal of blood equal to 5% of body weight, the survival rate attained 100%. When the lost blood had amounted to 6% of body weight, 6 of 7 pigeons survived; the one which did not survive exhibited marked seizures and autopsy revealed accumulation of serous fluid in the pericardial sac. The effect of bleeding amounting to 7% of body weight was studied in 11 pigeons. In this group, one of the animals died while subjected to haemorrhage, exhibiting seizures, and further two pigeons

Table I

Survival of unanaesthetized pigeons after loss of 5 to 8 ml blood per 100 g body weight

Serial No.	Body weight	Bleeding							Note
		First		Interval	Second		Total		
		min	ml	min	min	ml	ml	ml/100 g	
35	390	35	13.3	60	40	6.0	19.3	4.94	+
29	310	15	9.2	70	15	6.3	15.5	5.00	+
30	445	40	11.4	65	20	10.9	22.3	5.00	+
31	345	15	11.2	60	10	6.1	17.3	5.00	+
32	355	45	11.2	60	30	6.7	17.9	5.04	+
33	410	45	15.0	75	20	6.5	20.5	5.00	+
34	355	35	11.0	75	40	6.8	17.8	5.00	+
172*	495	continuously for 93 min					25.0	5.05	+
36	450	25	12.8	95	80	14.2	27.0	6.00	+
37	420	75	17.3	160	8	7.9	25.2	6.00	+
39	390	70	15.7	60	65	7.7	23.4	6.00	+
38	375	15	11.5	90	30	11.3	22.8	6.08	+
43	360	30	14.5	60	45	8.2	22.7	6.30	§
173*	330	10	9.9	30	12	9.9	19.8	6.00	—
176*	400	10	12.0	20	10	12.2	24.2	6.05	=
45	390	37	15.7	65	43	11.7	27.4	7.02	□
46	412	27	16.6	60	25	12.5	29.1	7.06	+
47	425	34	17.2	65	45	12.7	29.9	7.02	+
51	400	22	16.0	68	55	12.2	28.2	7.05	□
52	387	30	15.5	60	42	11.7	27.2	7.02	—
53	430	54	17.4	70	36	13.2	30.6	7.11	□
90	370	36	18.5	100	30	7.4	25.9	7.00	□
80	350	20	14.0	90	30	10.5	24.5	7.00	§
40	400	45	16.1	55	35	12.1	28.2	7.05	o
41	480	36	19.2	70	35	14.5	33.7	7.02	o
88	330	45	16.5	90	45	5.6	22.1	6.69	+
42	402	—	—	—	—	—	31.0	7.71	□
86	320	35	16.0	90	30	9.6	25.6	8.00	+
79	390	30	15.6	90	45	15.6	31.2	8.00	+
108*	345	15	13.8	90	15	13.8	27.6	8.00	§

+ Survived, observation for 7 days
□ Survived, observation for 3 to 5 days
= Survived, observation for 24 to 48 hours
§ Hypermotility and seizures during bleeding which led to acute death
o Died during or after flying
* Anaesthetized pigeons

Table II

Respiratory rate in pigeons before and after bleeding in response to forced flying

Before bleeding				After bleeding of 7% b.w.			
No.	Before	During	After	No.	Before	During	After
	forced flying (1 min)				forced flying (1 min)		
	Respiratory rate per min				Respiratory rate per min		
44	26	48	26 (10 min)	40	26	80	40 (60 min)
43	14	44	14 (6 min)	41	56	206	64 (60 min)
45	22	72	22 (9 min)	42	26	100	28 (30 min)
46	26	52	29 (9 min)	45	18	62	22 (15 min)
47	18	56	18 (8 min)	46	18	66	20 (15 min)
51	32	50	27 (5 min)	47	16	54	18 (15 min)
52	24	54	24 (5 min)	51	18	70	20 (25 min)
				52	22	56	22 (20 min)
				53	15	62	18 (20 min)
Mean:	23.1	53.7	22.8 (7 min 25 sec)		23.9	84.0	28.0 (29 min)

Figures in brackets mean the point of time of the second measurement of respiratory rate after forced flying

died during and after forced flying. Thus, of 10 pigeons subjected to severe haemorrhage and then to muscular exertion 8 survived. When 8% of body weight had been removed, 3 pigeons out of 4 survived; the one dying in the course of the experimental procedure exhibited hypermotility then seizures.

As shown in *Table II*, the increase in respiratory rate in response to forced flying was different in the control group and in pigeons subjected to haemorrhage. During the one-minute flight, the control animals showed more vigorous wing-flaps, and the increase in respiratory rate was smaller than in the group subjected to bleeding. The initial mean respiratory rate (23.1/min) increased to a maximum value of 53.7, and returned to the control value within 10 min. In contrast, after haemorrhage amounting to 7% of body weight respiratory rate increased from an average of 23.9/min to 84/min, and the initial value was attained only after 29 min. Two pigeons in which the respiratory rate failed to return to the resting value even after 1 hour, died. One of the 10 animals died during flying.

The changes in arterial blood pressure in response to bleeding (5 and 6 ml/100 g of body weight) are shown in *Table III*. These pigeons received urethane. Measurement of blood pressure in 59 normal pigeons revealed an average value of 101 $\pm$ 7 mm Hg. In response to the first bleeding in the group where

Table III

Blood pressure in anaesthetized pigeons after haemorrhage

No.	Volume of blood lost (per cent of b.w.)	Blood pressure in mm Hg						
		Before	At the end of	30 min after	At the end of	1 to 2 hours after	2 to 3 hours after	3 to 4 hours after
		First bleeding			Second bleeding			
121	5	80	46	58	38	45	—	60
126	5	125	80	78	60	—	—	—
133	5	110	90	100	56	75	—	—
134	5	110	70	85	50	—	80	—
135	5	70	50	80	40	—	—	60
138	5	107	78	82	56	—	—	72
139	5	95	75	75	56	—	66	—
140	5	115	65	78	38	—	—	47
141	5	95	62	82	50	—	—	73
	Mean:	101	68	80	49	60	73	62
143	6	137	73	90	40	48	60	—
144	6	78	45	52	30	—	29	28
145	6	120	80	96	60	81	—	80
146	6	72	44	50	30	43	56	—
147	6	105	84	98	60	—	—	90
170	6	116	48	53	44	61	62	63
173	6	106	56	62	37	—	—	70
178	6	98	68	74	40	—	68	—
	Mean:	104	62	72	43	58	55	66

5% of body weight had been removed, blood pressure fell to 68 mm Hg, and it was partially restored during the interval of 30 min. The second haemorrhage reduced blood pressure to 49 mm Hg. After cessation of bleeding a certain restoration was again observed, and without fluid replacement blood pressure increased to 62 mm Hg after 3 to 4 hours. Essentially the same results were obtained in the group where the volume of blood removed was 6% of body weight *(Table III)*.

Cardiac output in 22 normal pigeons attained 78.2 $\pm$ 59 ml/100 g on the average, which is about two to three times the values found in rats. After haemorrhage amounting to 5 to 6% of body weight, cardiac output decreased to 41.1 $\pm$ 33 ml/100 g; the difference between the control and the experimental group was statistically significant.

Total peripheral resistance in the normal pigeon was $11.6 \pm 0.9 \times 10^3$ dyne $sec \cdot cm^{-5}$, whereas 3 to 4 hours after haemorrhage an average of $12.8 \pm \pm 0.9 \times 10^3$ dyne $sec \cdot cm^{-5}$ was obtained.

Oxygen consumption in normovolaemic anaesthetized pigeons attained in 8 experiments was 18.4 ml/kg/min, and this was reduced by haemorrhage to 13.4 ml/kg/min, to increase to 15.9 ml in 4 hours. It was difficult to interpret the metabolic rate as the animals exhibited different movements. Nevertheless, the results obtained were sufficient to estimate the degree of oxygen deficiency.

Discussion

The maximal bleeding that can be tolerated but already leads to irreversible damage is 3.9% of body weight in the rat, 4.3% in the cat, and 4.7% in the dog. According to the present observations, pigeons tolerate graded haemorrhage much better, and most of them survive. In some experiments we succeeded in removing within 2 hours the whole amount of circulating blood volume as calculated from separate blood volume measurements, and still these pigeons survived.

Different explanations can be taken into consideration to account for the difference between the pigeon and the mammals. The most plausible one seems to be the difference in blood volume between avian and mammalian species. The degree of blood loss tolerated by birds and mammals, however, shows a greater difference than the difference in circulating blood volume. Thus, other factors have also to be considered.

Arterial blood pressure and cardiac output were considerably reduced after bleeding and thus the possibility arises that hypoxic tissue damage must have been substantial in these animals. However, after the removal of 6% of body weight of blood, in the pigeons cardiac output was about the same as that found in rats in the resting state. It seems therefore reasonable to assume that in the pigeon hypoxia is of a considerably smaller degree after haemorrhage, and the oxygen supply of vital organs may be sufficient to maintain a certain level of metabolism. In vertebrates the oxygen stores are extremely small and during apnea for 1 or 2 min they are fully exhausted. This holds also for the diving vertebrates, and the possibility to remain under water for longer periods of time can be explained first of all by the redistribution of circulating blood, cerebral and myocardial oxygen supply remaining essentially unchanged while the other organs receive practically no blood. It is an interesting observation that during diving oxygen consumption decreases to very low values (ANDERSEN 1959, 1961). It is known that the arterial pO_2 of diving birds, *e.g.* the duck, remains 25 to 30 mm Hg even after 5 to 8 min of diving, although arterial O_2 saturation falls below 5%. This is due to the concomitant acidification of blood,

the pH being depressed to 7.1 or 7.0. Because of the high pO_2 the animal does not lose its consciousness and is capable of consuming the entire amount of stored oxygen (ANDERSEN and LØVØ 1967).

According to previous observations from this laboratory the irreversibility of shock in mammals goes parallel with the severity of damage to the central nervous system (KOVÁCH and FONYÓ 1960; KOVÁCH 1961). Also, oxygen consumption of the brain increases (KOVÁCH *et al.* 1957, 1959) whereas its pO_2 decreases significantly (KOVÁCH *et al.* 1965). The difference in susceptibility to haemorrhage between birds and mammals might be due to the better oxygen supply of the brain in avian species or the diminished sensitivity to hypoxia. This is certainly a possible explanation in the case of flying and diving birds considering the extraordinary conditions of life and the endangerment of cerebral blood supply impending in these species.

Oxygen consumption of the pigeon has been found, in agreement with the results of GROEBBELS (1928), to be considerably higher than that of mammals of about the same body surface. Remarkably, despite a marked reduction, O_2 consumption remained high after haemorrhage. This might have been due to the increased motility of some of the animals in the course of the experiments. In view of the oxygen deficit theory of GUYTON and CROWELL (1961) it is possible that during haemorrhage the deficit of oxygen consumption is smaller in the pigeon than in mammals. According to the present investigations O_2 consumption in the pigeon is not reduced to such low values during haemorrhage than in the duck during diving (ANDERSEN 1961).

Muscular exertion (forced flying) in the pigeons subjected to haemorrhage caused death in 2 of the 10 birds, and in the remaining 8 pigeons regeneration was much slower than in the other group. This observation may indicate that after the loss of a similar amount of blood the hypoxic damage, particularly that to the brain, is smaller in the pigeon than in mammals. During exertion, however, hypoxia may attain a considerable degree owing to the shifting of blood towards the muscles.

The resistance of the pigeon to blood loss may also be due to a more efficient regulation of blood volume by the different compensatory mechanisms. It is possible that the blood stores are more efficiently emptied and/or a haemodilution occurs due to the transfer of fluid across the cell and capillary membranes from the intracellular and interstitial spaces into the vascular bed. Some haemodilution in response to blood loss has been described to occur also in mammals (ADOLPH *et al.* 1933; MELLANDER 1963). As to the neural control of fluid transport under such circumstances the mechanisms described by ÖBERG (1964) may be operative.

It has been shown by FOLKOW *et al.* (1966) that the capillary filtration coefficient (C.F.C.) is higher in the duck than in mammals, while in the turkey the values are similar to those found in mammals. The possibility thus arises

that there is some causal relationship between the degree of adaptation to flying, eventually also to diving, and the ability of the bird to regulate its body fluid compartments.

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ELECTROENCEPHALOGRAPHIC STUDIES OF THE HABITUATION IN HUMANS

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Repetition of tone or flashing light led to the synchronization of EEG activity in response to testing stimuli after 50 to 200 presentations. The first appearance of synchronization was observed in the temporo-parietal recordings of the dominant hemisphere. Volitional motor activity like hand-grasp did not interfere with the development of the synchronized EEG response. Also, memorization of simple tasks did not disturb the synchronization due to the presentation of the testing stimulus. After pairing of a volitional motor activity with repetition of a tone, synchronization frequently preceded the motor response. Repetition of the volitional motor reaction in the absence of a testing stimulus also resulted in synchronization of EEG activity. The possible physiological significance and the neurophysiological basis of the observed electrocortical response are discussed.

Habituation of behavioural and electrophysiological arousal reactions to repetition of a novel stimulus, and the biological significance of this phenomenon in learning processes have been studied by numerous investigators (Dodge 1923; Pavlov 1928; Thorpe 1950; Livanov and Poliakov 1945; Hernandéz-Péon 1960; *etc.*). On the basis of animal experiments the stage of habituation was indicated by the disappearance of behavioural and vegetative reactions and by a loss of EEG desynchronization in the course of the repetition of a novel stimulus.

Association of an unconditional stimulus with a neutral environmental signal until development of temporary connections led to a stage in which the conditional signal alone sufficed to elicit a synchronization of EEG activity (Morrell 1958). The physiological meaning of this synchronization in the advanced stage of conditioning was analyzed by Yoshii (1956).

Repetition of acoustic or visual stimuli may evoke synchronization of EEG activity in humans; the conditions necessary for the development of this phenomenon have been studied in the present investigation.

Methods

Electroencephalographic recordings were carried out on 16 human subjects of both sexes, ranging in age between 18 and 58 years, in more than 300 daily sessions. Both EEG and electromyographic recordings were performed through skin electrodes with a Hellige 12-channel Neuroscript apparatus.

All subjects were mentally normal and engaged in some kind of daily work. A daily session lasted 60 min, usually between 4 and 7 p.m.; it took place in a comfortable position in a sound-proof room and the light was off only during sessions when flashing light was used as a testing stimulus. A two-way loudspeaker and a one-way mirror window served for communication between the subject and the investigators.

Electroencephalographic activity was recorded from the frontal, parietal, temporal and occipital cortices, with ipsi-hemispheric and interhemispheric leads. Electromyographic recording was carried out by means of two skin electrodes placed at a distance of 2 cm on the radial side of the hand.

Tone and flashing light frequency was triggered with a square-wave impulse generator; 4 and 12 cps stimuli were used in the routine procedures. Tone intensity could be changed by an amplifier connected between the generator and the loudspeaker.

Results

Repetition of a 4 or 12 cps tone in 40 sec intervals was accompanied by a desynchronization of EEG activity in the first daily session and mostly during the first 10—15 trials. The testing stimulus usually lasted for 10 seconds but on some occasions it was reduced to 6 and/or 8 seconds. In each daily session the subjects were exposed to 50 signals and in most cases presentation of the testing stimulus did not evoke desynchronization at the end of the first session. After presentation of 100 to 150 trials high amplitude slow waves were induced on the temporo-parietal cortex. This synchronized activity appeared at the frequency range of 8 to 11 cps, although in some occasions high amplitude discharges of 5 to 6 cps characterized the response. There was no relation between the frequency of the testing stimulus and the frequency of synchronization. The use of 4 and 12 cps tones which were presented alternately in 40 sec intervals led to synchronization of an identical frequency range. The hemisphere dominant on the basis of handedness showed the response with greater amplitude and in some cases hardly any synchronization was detected in the non-dominant hemisphere. After several hundred trials the synchronization spread over the whole cortex and high amplitude slow waves could be recorded from both frontal and occipital areas (*Figs 1* and *2*).

Repetition of the flashing light at 4 or 12 cps frequency also led to synchronized EEG response after 100 to 150 trials. In preliminary studies we found that a high illumination intensity prevented the development of a stable response, therefore the intensity was decreased by moving the light source away from the subject, to achieve a reflected rather than a direct retinal stimulation.

After a synchronized EEG response to the testing stimulus had developed, further trials over more than 40 daily sessions did not modify its character. Some weakening or augmentation of the response could be observed for one or two days, but there was no tendency for its diminution during the observation period. A sudden change in tone intensity, or changes in the intertrial intervals ranging from 5 to 120 sec did not influence the response or at most induced its transitory decrease for one or two trials.

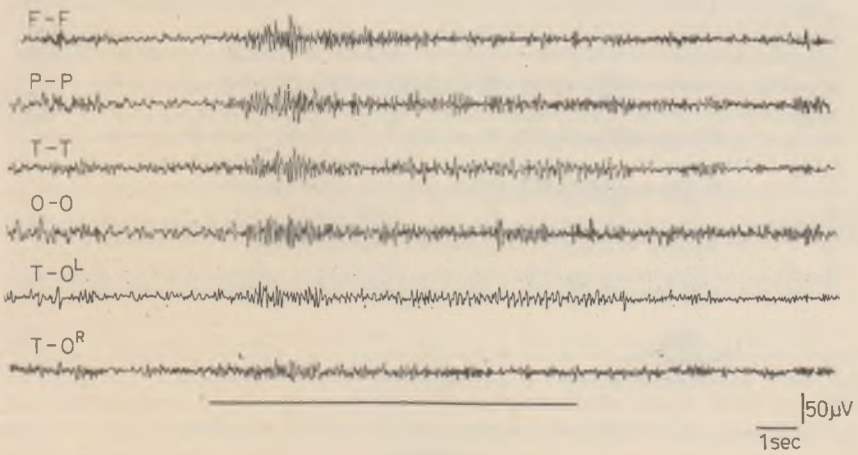


Fig. 1. Synchronized slow waves in response to tone after a number of repetitions of testing stimulus. Horizontal line indicates duration of stimulus

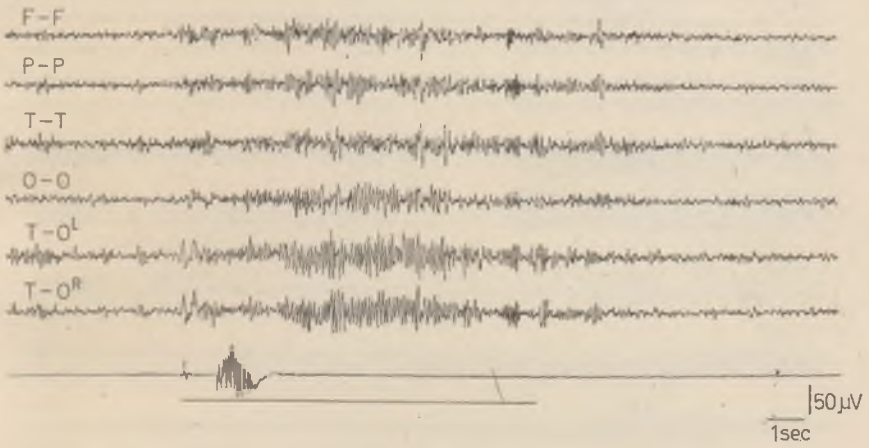


Fig. 2. Synchronization of EEG activity induced by flashing light as the result of repetition of testing stimulus

In further observations the persons were requested to perform a hand grasp lasting for one or two sec on the presentation of testing stimulus. After a few associations of this volitional motor reaction with the testing stimulus, a synchronized EEG response developed frequently preceding the motor response *(Fig. 3)*.

The testing stimulus was presented in 40 sec intervals and the persons were requested to repeat the hand grasp reaction in the absence of the testing stimulus in the intertrial intervals. They produced this secondary response 10 to 15 sec following the previous testing signal. The motor reaction was accompanied by a synchronized EEG activity of a character similar to that observed in response to the testing stimulus *(Fig. 4)*.

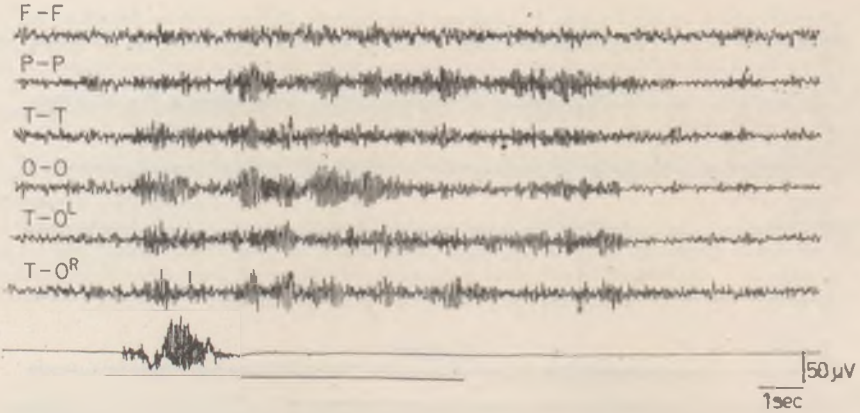


Fig. 3. Synchronization of EEG activity induced by tone accompanied by electromyographic response

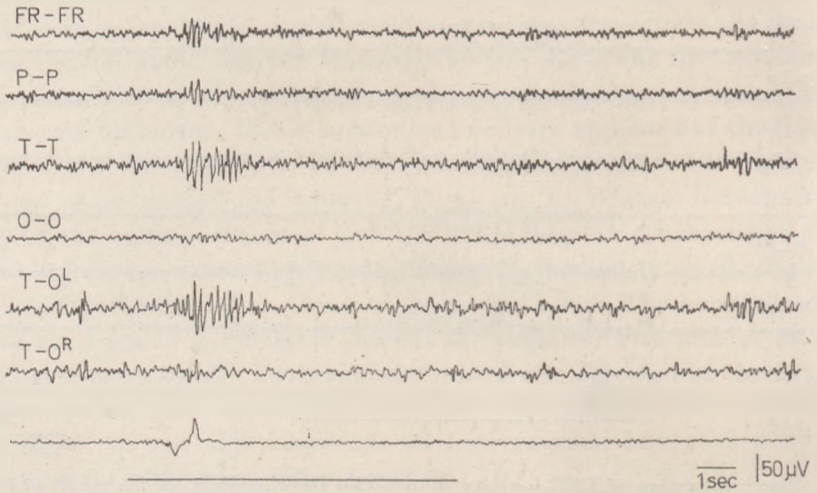


Fig. 4. Slow waves of 5 to 6 cps evoked by tone after repetition of testing stimulus. Note high amplitude waves in the temporal regions on the dominant hemisphere

In other experiments the persons received ten testing stimuli and were requested to produce hand grasp reactions in approximately the same intervals in which the testing stimuli were presented (15 sec intervals were used). During this period they did not receive testing signals and performed the motor reaction according to their time estimation. The motor activity was accompanied by synchronized EEG activity and usually the appearance of high amplitude slow waves was preceding the motor performance *(Fig. 5)*.

Simple mental tasks such as counting or memorization of words which were requested in the intertrial intervals and called for answer after a few trials,

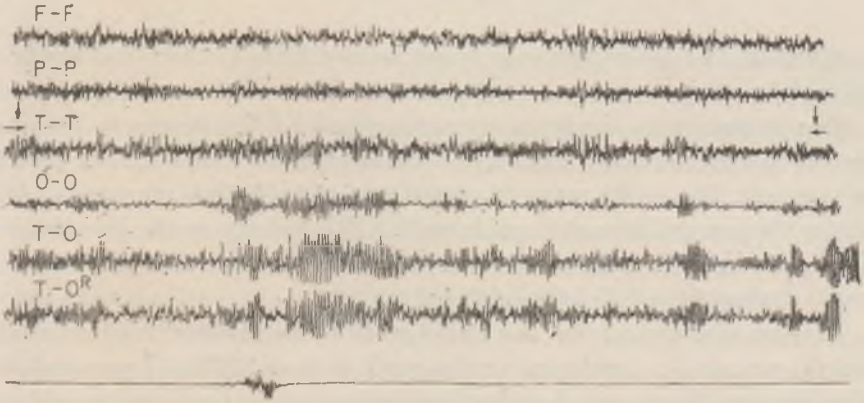
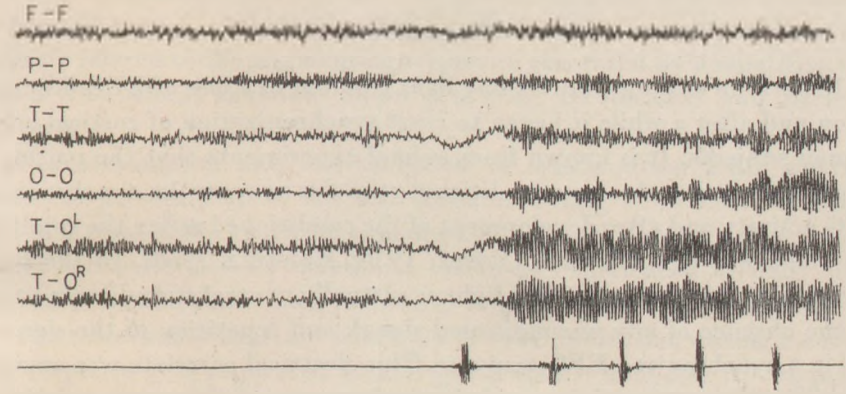
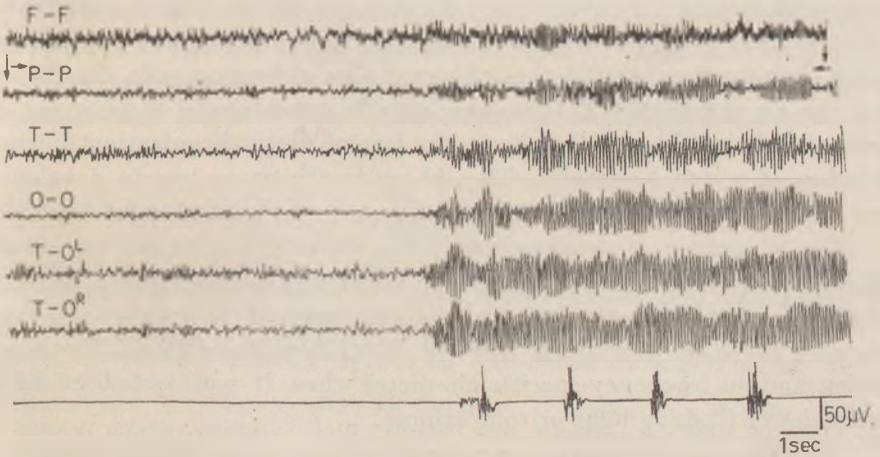


Fig. 5. Volitional hand-grasp reaction accompanied by synchronization of EEG activity. Note marked response in the temporal and less in the occipital leads. Motor response was performed in the intertrial interval (see in text)



(A)



(B)

Fig. 6. Synchronization evoked by click in the course of the following of tone by hand-grasp reactions. Clicks were given in 3 sec intervals. First series (A) of click presentation resulted in temporary desynchronization. Repetition of the series during the same session led to prompt synchronization (B)

did not disturb the development of synchronized EEG activity due to the testing stimulus.

Two persons who displayed a synchronized EEG response to the presentation of a tone were requested to follow clicks given in 3 sec intervals by a hand grasp reaction. The clicks were presented in series of 40 to 60 signals. After a few series the persons began to synchronize EEG activity on the presentation of the first click before the first motor response. High amplitude slow waves were observed during the whole series *(Fig. 6)*.

In view of the subjects' sex and age differences, some relation could be expected between the development of a synchronized EEG response and the individual habits. There was, however, no indication that any sex would be superior in this respect or some age group would tend to develop the response.

Discussion

Repetition of a tone or flashing light resulted in a loss of EEG desynchronization and after a while it began to elicit synchronization of cortical activity in human subjects. It is known from animal experiments that the pairing of a conditional signal with an unconditional stimulus may evoke synchronization of EEG activity and after development of the conditioned reflex the conditional signal alone was also effective (Yoshii 1956; Morrell 1958). In the present investigations a tone or a flashing light as virtually neutral stimuli were presented in the absence of any unconditional signal, and repetition of the signals resulted in a synchronized EEG response. This electrical correlate was associated with volitional motor activity, and the performance of the motor reaction was also accompanied by the appearance of synchronization. It seems that the synchronized EEG response is a special form of temporary connections in which neither the situation nor the applied signal play a decisive role. On the other hand, the synchronized EEG response may be considered a third stage in the course of habituation, which follows the loss of desynchronization during the repetition of a novel stimulus. This phase is absent or poorly developed in lower vertebrates, nevertheless the final answer needs further information.

The frequency of synchronized EEG activity due to the test-stimulus ranged between 8 and 11 cps, and was unrelated to the frequency of the applied stimulus. In three cases the frequency of synchronized slow waves was ranging between 5 and 6 cps in response to either 4 or 12 cps test-stimuli. The synchronization had no frequency-specific character when it was tested at different frequencies of flashing light or tone stimuli.

The first establishment of synchronized slow waves in the temporal and parietal regions was common in response to either light or tone stimuli, and the occipital leads gave almost flat records during hypersynchronization of the former areas. Bursting alpha-waves could be observed during intertrial intervals.

According to the theories concerning the nature of habituation, this phenomenon might be attributed to a particular central nervous "centre", a kind of cerebral censor which somehow controls the sensory inputs and sends out inhibitory commands to the relay stations through which the unwanted signals must pass (SHARPLESS 1964; JOUVET 1963; GRIFFIN 1963; *etc.*). JOUVET (1963) presented evidence indicating that the arousal reaction does not habituate readily in decorticate animals. In contrast, habituation was observed to occur in spinal animals (PROSSER and HUNTER 1936; KOZAK *et al.* 1962).

The present observations have made us to assume that habituation cannot be considered as a merely centripetal inhibition of sensory input. The appearance of synchronized slow waves after a number of presentations of the test stimulus supports the view that habituation is an active process which manifests itself with corticifugal actions. The synchronization may be attributed to recruiting responses of thalamocortical circuits. Spindle waves of 8 to 12 cps are closely related to the augmenting responses generated by a repetitive stimulation of the lateral thalamic nuclei (LI 1956; BROOKHART and ZANCHETTI 1956; *etc.*).

The attenuation and augmentation of synchronized EEG activity in response to presentation of the test-stimulus during one session or within a period of a several days may be explained by the influence of either mental or physical activities and by emotional reactions experienced in daily life, although the mechanism of such influences requires further investigations.

The remarkably constant character of synchronization during a volitional motor response or a requested mental performance indicates its organization independent from the neurophysiological mechanisms involved in psychic events. Furthermore, the data clearly showed that the synchronization can be organized in the absence of an appropriate sensory input and elicited by a volitional motor response. The phenomenon may be attributed to the temporary connection which has developed between the signal-induced synchronization and the volitional motor reaction.

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Pharmacologia

TISSUE DISTRIBUTION OF CHLOROQUINE IN THE RAT

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The concentration of chloroquine in the rat's blood plasma, liver, spleen, lungs, kidneys, heart, skeletal muscle, brain and testes was estimated after the oral administration of 20, 40, 80 and 400 mg/kg of the drug.

By increasing the dose, the concentration of chloroquine rose more markedly in the liver, heart and skeletal muscle than in other organs, and larger fraction of the administered amount of chloroquine was retained in the rat's organism. Similar results were obtained after daily administration of 20, 40, 80 and 160 mg/kg for one week. After cessation of treatment with daily 160 mg/kg the concentration of chloroquine in the liver, cardiac and skeletal muscle decreased more rapidly than in other organs. This was not observed with doses of 20 and 40 mg/kg/day. Thus, the ratio of chloroquine concentrations in the organs depends on whether non-toxic or sublethal doses have been administered. The possible causes of the difference are discussed.

Experimental (BERLINER *et al.* 1948; MCCHESNEY *et al.* 1965) as well as clinical observations (BÄUMER *et al.* 1959; RUBIN *et al.* 1963) have indicated that chloroquine when given for longer periods of time, is readily accumulated in the body. Data concerning the relationship between storage and the administered dose are, however, lacking. According to MCCHESNEY *et al.* (1967), after the administration of chloroquine with food for 7 months approximately the same fraction of the dose was stored in the rat when 1.9 mg/kg or 16.8 mg/kg had been given daily. On the other hand, in a previous study we found that of sublethal doses more was stored in the liver than of lower doses corresponding to the amounts given by MCCHESNEY *et al.* (1967). Experiments were therefore performed to establish whether in the rat the storage of chloroquine in the organs was proportional to the dose.

Methods

Experiments were performed on male albino rats weighing 180 to 220 g kept on a standard diet. Five hours prior to the administration of chloroquine food was withheld but free access to water was allowed. Chloroquine diphosphate was dissolved in distilled water and given by stomach tube. The doses as well as the concentrations in plasma and the various organs were computed for the free base. The drug was given either in one or more daily doses

for one week. In the second series of experiments the ingested amount of food and water and the pH of the urine were recorded daily.

Subsequently under superficial ether anaesthesia the animals were bled from the abdominal aorta. The blood was immediately heparinized and centrifuged. Plasma chloroquine concentration was estimated in the samples, while in the group treated with 20 mg/kg of chloroquine the drug was estimated in the pooled samples of the same volume obtained from two rats. The organs were weighed and homogenized in a Potter blendor with distilled water to ensure a 2 to 10% concentration according to the expected chloroquine level.

Chloroquine was estimated in plasma and organs by the method of McChesney *et al.* (1962); in some experiments, the result was checked by the paper chromatographic technique of Kuroda (1962). The amount of chloroquine metabolites in the extracts was below 10% of that of the parent compound.

For histological studies the sections were fixed in formalin, embedded in paraffin, and stained with haematoxylin-eosin.

Results

Table I shows the concentration of chloroquine in plasma and various tissues 24 hours after a single oral dose. By increasing the dose of chloroquine from 20 to 400 mg/kg, concentration rose more rapidly in the liver, heart and skeletal muscle than in other tissues. This was clearly shown by the ratio tissue concentration/plasma concentration which was about twice or three times higher in the liver, heart and skeletal muscle after the administration of 400 mg/kg than after giving 20 mg/kg. In the other organs the ratio did not change so uniformly and to such an extent.

By increasing the dose a successively greater fraction of the administered chloroquine was retained. The majority was found in the skeletal muscles and the liver which amount to about half of the animal's body weight. Since the

Table I

Tissue concentration of chloroquine in rats 24 hours

Dose, mg/kg	Number of animals	Tissue concen-			
		Tissue/plasma			
		Plasma	Liver	Spleen	Lung
20	8	0.10 ± 0.03	46 ± 6 460	66 ± 8 660	47 ± 3 470
40	10	0.22 ± 0.03	135 ± 26 613	146 ± 32 668	98 ± 17 445
80	8	0.34 ± 0.07	285 ± 38 838	215 ± 42 633	192 ± 31 565
400	12	1.42 ± 0.16	1940 ± 180 1366	1020 ± 74 718	680 ± 55 479

[1] Mean value ± standard deviation. Values are based on wet weight of tissue.
[2] As percentage of the dose. Muscle is assumed to amount to 45.5% of body weight

spleen, lungs, kidneys, heart, brain and testes together attain about 5% of the body weight, the absolute amount of chloroquine retained in these organs was not significant, despite the relatively high tissue concentrations. In perirenal fat and in the skin chloroquine concentration amounted to 5 and 15 times the plasma concentration. According to CASTER *et al.* (1956), the total weight of the skin and fat tissue attains about 25% of the body weight. Thus, owing to the low concentration of chloroquine in these organs, the absolute amount accumulated in the skin and fat was negligible.

According to observations from this laboratory, the oral LD_{50} of chloroquine in the rat is 675 mg/kg (VARGA 1966). Of the 13 rats given 400 mg/kg one died, and in none of these rats was any pathological change revealed by the histological study.

The dose dependence of chloroquine distribution was apparent also after the administration of repeated doses *(Table II)*. Chloroquine storage was again most pronounced in heart muscle, liver and skeletal muscle. One day after the last dose of 20 mg/kg, 5.45% of the total amount of 140 mg/kg was retained, whereas after prolonged administration of 160 mg/kg, 15.6% was found to have been stored.

Of the 40 rats treated with 160 mg/kg daily, 4 animals died before the last day of treatment. In these rats and in 6 of the remaining 36 animals the histological study revealed centrilobular hepatic necrosis involving about 3 to 6% of the lobule. The heart and the skeletal muscles also contained focal necroses, and there was a moderate testicular atrophy. Other organs showed no pathological changes after the said dose. Of the 8 rats receiving 80 mg/kg of chloroquine

after oral administration of a single dose

tration mg/kg)[1] concentration ratio					Total retention in eight organs, per cent[2]
Kidney	Heart	Muscle	Brain	Testis	
30 ± 10 300	9.2 ± 2.1 92	2.2 ± 0.9 22	3.2 ± 1.1 32	4.0 ± 0.7 40	17.5
45 ± 9 205	24 ± 8 109	6.9 ± 1.4 31	4.7 ± 1.0 21	10 ± 3.1 45	24.8
107 ± 12 314	72 ± 8 212	16 ± 3.1 47	8.8 ± 2.1 26	17 ± 2.3 50	27.6
362 ± 33 255	330 ± 22 232	71 ± 13 50	35 ± 4 24	63 ± 6 44	30.1

(CASTER *et al.*, 1956).

Table II

Tissue concentration of chloroquine in rats after daily

Dosage mg/kg/day	Number of animals	Tissue concen-			
		Tissue/plasma			
		Plasma	Liver	Spleen	Lung
7×20	6	0.17 ± 0.04	112 ± 16 659	125 ± 23 735	104 ± 18 612
7×40	6	0.37 ± 0.10	274 ± 25 740	253 ± 19 683	225 ± 35 608
7×80	8	0.63 ± 0.17	890 ± 86 1412	635 ± 78 1008	587 ± 46 932
7×160	8	1.35 ± 0.28	2345 ± 270 1737	1320 ± 185 977	1160 ± 155 859

[1] Mean value ± standard deviation. Values are based on wet weight of tissue. Determina-
[2] As percentage of total dose. Muscle is assumed to amount to 45.5% of body weight.

one exhibited slight centrilobular hepatic necrosis. Lower doses caused no pathological change.

During prolonged treatment, the amount of food ingested was on the average 18% lower than in the week preceding the treatment. There was no statistically significant difference in the ingested amount of food between the various groups. In all probability, the reduction in food intake was due to the experimental procedure rather than to the toxic effect of the drug. Water intake was essentially unchanged during treatment.

Table III

Tissue concentration of chloroquine in rats after

(160 mg/kg/day,

Hours after last dose	Number of animals	Tissue concen-			
		Tissue/plasma			
		Plasma	Liver	Spleen	Lung
24	12	1.22 ± 0.23	2630 ± 240 2155	1410 ± 180 1155	1030 ± 110 844
48	8	0.68 ± 0.09	975 ± 110 1433	810 ± 88 1191	542 ± 75 797
96	8	0.23 ± 0.06	114 ± 23 495	195 ± 34 848	196 ± 22 852

[1] Mean value ± standard deviation. Values are based on wet weight of tissue.
[2] As percentage of total dose (7×160 mg/kg). Muscle is assumed to amount to 45.5%

oral administration of the drug, for a week

tration (mg/kg)[1] / concentration ratio					Total retention in eight organs, per cent[2]
Kidney	Heart	Muscle	Brain	Testis	
70 ± 12 412	23 ± 7 135	7.8 ± 3.0 46	7.0 ± 2.0 41	13 ± 3.5 76	5.45
132 ± 21 357	62 ± 5 167	20 ± 4.1 54	14 ± 3.1 38	43 ± 6.7 116	8.41
330 ± 27 525	195 ± 23 310	65 ± 12 103	23 ± 8.8 37	76 ± 6.2 120	12.6
720 ± 92 533	667 ± 50 494	140 ± 28 103	45 ± 6.0 33	150 ± 22 111	15.6

tions were performed 24 hours after last dose.

After the administration of large doses of chloroquine (160 mg/kg daily for a week) the concentration of the drug in the liver, heart and the skeletal muscles diminished more rapidly than in other organs *(Table III)*. One day after the administration of the last dose the hepatic chloroquine concentration was about 2 and a half times higher than in the lungs while on the fourth day it was already lower. A similar reduction could be observed in the heart and in skeletal muscle. When giving smaller doses (40 and 20 mg/kg for 7 days) the

stopping the administration of the drug

orally, for a week)

tration (mg/kg)[1] / concentration ratio					Total retention in eight organs, per cent[2]
Kidney	Heart	Muscle	Brain	Testis	
712 ± 85 584	585 ± 48 480	120 ± 18 98	52 ± 11 42	210 ± 32 172	15.8
325 ± 41 477	220 ± 30 324	52 ± 17 76	23 ± 8 34	130 ± 22 191	6.4
103 ± 10 448	25 ± 8 108	7.3 ± 3.2 32	8.0 ± 4.1 35	35 ± 14 152	0.92

of body weight.

diminution of chloroquine concentration in the said three organs was about the same as in the other organs.

Thus, chloroquine storage in liver, heart and skeletal muscle is enhanced when sublethal doses are administered but after cessation of the treatment the drug is more rapidly cleared from the above tissues than from the other ones.

Neither the present experiments nor the data of other authors provide a satisfactory explanation why the said organs accumulate substantially more chloroquine during treatment with high doses than the other organs.

Discussion

The present results indicate that the distribution of chloroquine in the body and the ratio of the concentrations of chloroquine in the different tissues depend, among others, on the employed dose. On increasing the dose, chloroquine levels tend to rise more rapidly in the liver, heart and skeletal muscles than in other tissues, and a continuously growing fraction of the administered amount of chloroquine is retained in the body. This observation is inconsistent with the findings of McCHESNEY *et al.* (1967). The discrepancy may be explained by the much higher doses of chloroquine used in the present experiments.

The urinary excretion of weak organic bases and acids is known to depend, among others, on the pH of the urine (MILNE *et al.* 1958). It has also been shown that the excretion of chloroquine is enhanced when the urine is acidic, and it is lower, hence the drug is retained in the body, when an alkaline urine is excreted (JAILER *et al.* 1949). In the present experiments, the pH of the urine varied between 6.3 and 7.2 in the groups receiving higher and lower doses, respectively. Thus, the increased retention of the drug observed with the higher doses could not be attributed to an eventual shift of the urinary pH in the alkaline direction.

In the liver the ratio of chloroquine and its metabolites was approximately 10 after the high dose and 3 to 4 after the small one. It was therefore reasonable to assume that the increased retention after high doses was due limited ability of the liver to metabolize chloroquine and therefore the fraction to the accumulated in the body is growing as the dose is increased.

In chronic toxicity studies on rats FITZHUGH *et al.* (1948) found that, at a dose level of about 60 mg/kg/day of chloroquine the incidence of death and the severity and occurrence of histologically verified lesions were much higher than it could have been expected on the basis of results obtained with smaller doses. The most severe changes, particular focal necroses, were revealed in the heart, liver and skeletal muscle, where we also found necroses. Thus the increased storage of chloroquine seems to be related to the severity of histological lesions. The degree of tissue damage, however, does not simply depend on the concentration of chloroquine since the levels found in the spleen, lungs and kidneys

were higher than those in the muscles, in spite of there having been no histologically verifiable lesions in the former organs.

The subcellular distribution of chloroquine seems to depend on the applied dose. After the administration of small doses the largest fraction of chloroquine in the liver and kidney cells can be found in the mitochondria, whereas in the case of higher doses the plasma fractions contain a larger amount of the drug (VARGA 1968).

To our best knowledge phenylbutazone is the only drug the distribution of which in the body depends on the dose (HEISE 1956). Further investigations are necessary to establish whether the dose-dependent distribution is shown only by these two compounds or it is a general pharmacological phenomenon.

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INTRACELLULAR LOCALIZATION OF CHLOROQUINE IN THE LIVER AND KIDNEY OF THE RAT

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Rats were given varying amounts (13 to 360 mg/kg) of chloroquine orally and intracellular distribution of the drug in liver and kidney was studied 24 hours later. The specific binding of chloroquine (μg/mg nitrogen) after the administration of 13 mg/kg in the hepatic cell fractions was: homogenate 0.79; nucleus 0.60; mitochondrium 2.34; microsome 0.34; soluble fraction 0.20. After the administration of 360 mg/kg the values obtained were: homogenate 57.1; nucleus 38.8; mitochondrium 12.5; microsome 22.2; soluble fraction 112. The intracellular distribution of chloroquine in the kidney also depended on the applied dose, *i.e.* on tissue concentration. There was no relationship between the nucleic acid and chloroquine content of the various cell particles. The possible cause of the dose-dependent intracellular localization of chloroquine is discussed.

The intracellular localization of drugs shows considerable differences. After treatment of dogs with mepacrine *in vivo*, the majority of the drug in the liver is found in the nuclei (BRODIE and HOGBEN 1957). The alkaline dye cyanin-863 in the rat kidney is bound to the mitochondria (RENNICK *et al.* 1956). Of digitoxin, more than 90% in the heart muscle cells is located in the cytoplasm (SPRATT and OKITA 1958). N-acetyl-4-aminophenazone, on the other hand, is not bound to any of the cell particles and is distributed in the cell water (BRODIE *et al.* 1951). There are drugs the subcellular distribution of which is different in the different tissues; quinidine, for example, in heart muscle is located in the nuclei, whereas in the liver it is the microsomal and cytoplasmic fractions which contain the drug in the highest concentration (ARORA *et al.* 1965). The factors determining the subcellular localization of the different drugs are not sufficiently known, consequently, their exact mechanism of action, mode of elimination, and the toxicology of many drugs have not been fully clarified.

The action and biological conversion of many drugs depend, both quantitatively and qualitatively, on the dose applied (the problem has been surveyed by ARIËNS 1964). The present study deals with the intracellular localization of chloroquine; our first aim was to establish whether the subcellular distribution of chloroquine depended on the dosage.

Methods

Experiments were made on albino rats weighing 180 to 220 g. Prior to administration of the drug the animals were starved overnight but allowed free access to water. Chloroquine diphosphate was given in aqueous solution through a stomach tube. The animals were killed

by bleeding from the carotids, liver and kidneys were removed and weighed. With a 0.25 M solution of sucrose containing 0.05 M phosphate buffer adjusted to pH 7.4 a 10% homogenate was prepared in a Potter blendor centrifuged for 2 min at 200 g, the sediment which contained the red blood cells, intact cells, connective tissue, *etc.*, was discarded and the supernatant was used in the further study. Sedimentation was undertaken at 600 g for 5 min, the sediment was homogenized with sucrose and recentrifuged; the fraction thus obtained contained the nuclei. The mitochondrial fraction was prepared by pooling the supernatants and centrifuging for 20 min at 9,000 g. The supernatant was then recentrifuged at 40,000 g for 1 hour, to obtain the microsomal fraction. Centrifugation was undertaken with *Zugló Lh-412* and *Type 1957 Phywe* ultracentrifuges, at a temperature ranging between 0 and + 5 °C.

Total nitrogen content of the homogenate and the fractions was determined by the method of CLEGHORN and JENDRASSIK (1934), desoxyribonucleic acid and ribonucleic acid by the method of CERIOTTI (1955), and succinodehydrogenase activity according to UMBREIT *et al.* (1951). Taking the amounts found in the homogenate as 100, the following values were found in the different fractions.

Nuclear fraction:

total nitrogen, 14.2%
DNA, 96.5%
RNA, 10.1%
succinodehydrogenase, 15.2%

Mitochondrial fraction:

total nitrogen, 23.5%
DNA, less than 1%
RNA, 13.2%
succinodehydrogenase, 74.3%

Microsomal fraction:

total nitrogen, 24.2%
RNA, 56.3%
succinodehydrogenase, less than 3%

Soluble fraction:

total nitrogen, 39%
RNA, 14.2%

Fractionation of the kidney homogenate was performed according to SCHNEIDER and POTTER (1949). In every second animal the nitrogen content of the kidney homogenate and of the various fractions was determined. The total nitrogen content of the homogenate attained 23.8 mg/g; taking this as 100, the following values were obtained for the various fractions: nucleus, 20.8%; mitochondrium, 18.2%; microsome, 15.1%; soluble fraction, 45.9%.

Chloroquine content of the liver and kidney homogenates and the different fractions was estimated by the method of MCCHESNEY *et al.* (1962).

Dosage and tissue concentrations of chloroquine were computed for free base.

Results

The distribution of chloroquine in the various fractions of liver cells is shown in *Table I*. Twenty-four hours after the administration of 13 mg/kg of chloroquine almost 70% of the drug, whereas after the administration of 360 mg/kg, only 5.1% was found in the mitochondrial fraction. The opposite was the case with the soluble fraction: at low (24 μg/g) tissue concentration only 10% of the total chloroquine in the cells was found in the soluble fraction, whereas with 1740 μg/g tissue concentration the majority was in the soluble fraction. Irrespective of the concentration of chloroquine, 8.5 to 10.7% was found in the nuclear fraction and 9.4 to 15.3% in the microsomal one.

Table I

Intracellular localization of chloroquine in rat liver 24 hours after administration

Dose, mg/kg orally	Chloroquine, μg/g	Percentage of chloroquine recovered in subcellular fractions[1]			
		Nuclear	Mitochondrial	Microsomal	Soluble
13	24 ± 8[2]	10.7 ± 2.6	69.0 ± 13	10.3 ± 1.9	10.0 ± 2.5
40	103 ± 14	8.53 ± 1.8	60.0 ± 6	15.3 ± 4.2	16.2 ± 3.1
120	342 ± 45	9.10 ± 2.1	32.4 ± 5.2	15.0 ± 3.6	43.5 ± 4.2
360	1740 ± 350	9.62 ± 1.4	5.1 ± 2.1	9.4 ± 1.8	75.9 ± 9.2

[1] The amount of chloroquine recovered in four fractions is considered 100%.
[2] Mean values of five rats at each dose level ± standard deviations. Values are based on wet weight of liver.

The affinity of chloroquine to the mitochondrial fraction was apparent also when the chloroquine concentration of the various fractions was computed for nitrogen content. Calculated from the above figures, the specific binding of chloroquine, as expressed in μg/mg of nitrogen, attained the following values at a liver concentration of 24 μg/g: homogenate 0.79; nuclear fraction 0.60; mitochondrial fraction 2.34; microsomal fraction 0.34; soluble fraction 0.20.

Similar results were obtained when the localization of chloroquine in the liver cells was estimated 8 hours after administration, the period necessary for the complete absorption of chloroquine from the gastrointestinal tract (VARGA 1966).

Three hours after the intravenous administration of 25 mg/kg (four cases) the intracellular localization of chloroquine was practically identical with the distribution observed after the oral administration of 13 and 40 mg/kg, the nucleus containing 7.9%, the mitochondrial fraction 63.5%, the microsomal 14.7%, and the soluble fraction 13.9%. Thus, the intracellular distribution of chloroquine does not depend on the mode of administration.

When evaluating the above findings, it had to be taken into account that during the fractionation procedure chloroquine may have dissociated from the sites where it had been bound *in vivo*. To reduce the possibility of such a methodological error, in a control experiment only two fractions were separated. The homogenate was immediately centrifuged at 9,000 g for 20 min, and the chloroquine content was estimated in the sediment containing the nuclei and the mitochondria, as well as in the supernatant containing the microsomes and the cytoplasm. After the administration of 13 mg/kg in three experiments the nuclear—mitochondrial fraction contained 82.5% while the microsomal—soluble fraction 17.5% of the intracellular chloroquine. These values were in agreement with the figures presented in *Table I* (79.7 and 20.3%, respectively). After the oral administration of 360 mg/kg in three cases the nuclear—mitochondrial

Table II

Intracellular localization of chloroquine in rat kidney 24 hours after administration

Dose, mg/kg orally	Chloroquine, μg/g	Percentage of chloroquine recovered in subcellular fractions[1]			
		Nuclear	Mitochondrial	Microsomal	Soluble
40	41 ± 7[2]	14.6 ± 3.3	49.5 ± 5.2	17.8 ± 3.1	18.1 ± 3.8
120	127 ± 22	8.5 ± 2.1	27.2 ± 4.8	16.2 ± 2.4	48.1 ± 5.2
360	295 ± 38	10.3 ± 2.6	16.8 ± 3.4	14.0 ± 3.1	58.9 ± 4.5

[1] The amount of chloroquine recovered in four fractions is considered 100%.
[2] Mean values of five rats at each dose level ± standard deviations. Values are based on wet weight of kidney.

frac-tion contained 21.5% of the total cellular chloroquine and 78.5% of the amount present in the supernatant; the corresponding values in *Table I* are 14.7% and 85.3%, respectively. Thus, fractionation of the various cell particles must have led to some redistribution of the intracellular chloroquine, but this shifting was not of such a degree which could have substantially influenced the conclusions drawn from the first series of these experiments.

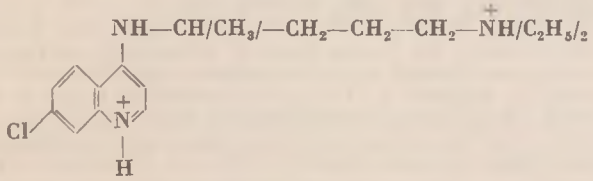
Table II shows the localization of chloroquine in the renal cells. At 41 μg/g tissue concentration about half of the intracellular amount, whereas at 295 μg/g tissue concentration only 17% was found in the mitochondrial fraction. With increasing tissue concentrations a growing fraction of chloroquine was found in the soluble fraction, similarly to the situation observed in the liver.

Discussion

Parker and Irvin (1952a) and Kurnick and Radcliffe (1962) have shown that chloroquine is readily bound to nucleic acids and it has been assumed that this binding determinates the intracellular distribution of the drug *in vivo*. The present results could not confirm this assumption since there was no relationship between the chloroquine and nucleic acid content of the various cell particles. Our findings are more in accord with the histological studies of Bäumer *et al.* (1959) who found that in the white blood cells of chloroquine treated rabbits the majority of the drug can be found in the cytoplasm whereas the nucleus contains only insignificant amounts.

It has also been shown that there is an equilibrium between $^{+}HB - B'H^{+} \rightleftarrows B - B'H^{+} + H^{+}$ (B indicates the nitrogen atom of the ring, and B′ the nitrogen of the diethylamino group of the side-chain). With increasing

pH the equilibrium is shifted towards the right and the fraction of chloroquine bound to plasma albumin ($P \cdot B - B'H^+$) increases (PARKER and IRVIN 1952).



In the rat's hepatic cells the highest pH values are found in the mitochondria (NOVELLI *et al.* 1962). This provides an explanation of the mitochondrial accumulation of chloroquine. The assumption is supported by the fact that the distribution *in vivo* of weak organic bases, thus also of chloroquine, is significantly affected by changes in the acid—base equilibrium (JAILER *et al.* 1948; RUBIN *et al.* 1965). The possibility cannot, however, be excluded, that the affinity of chloroquine to the mitochondria is due to the quinoline structure of the molecule. RENNICK *et al.* (1958) succeeded in demonstrating that many compounds with a quinoline structure such as the dye cyanin-863, are accumulated in the mitochondria of the renal cells.

In a previous paper (VARGA 1968) it has been shown that the distribution of chloroquine in the body depends on the applied dose. The present results indicate that this holds also for the intracellular distribution of the drug. If the tissue concentration is low the compound shows a great affinity to the mitochondria, and with increasing concentrations the intracellular localization is altered presumably because the mitochondria cannot take up more of the drug.

It is likely that a dose-dependent distribution is characteristic of many drugs and this has to be taken into account particularly with compounds which are used in a wide range of dosage and which are accumulated in the body.

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THE CENTRAL NERVOUS EFFECTS OF BIS-(4-AMINOPHENYL)-ALKYL DERIVATIVES

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It has been shown that γ,γ-bis-(4-aminophenyl)-propylamine (TK 174) does not penetrate into the central nervous system and has therefore no central effect in the rat. The N-methyl and N,N-dimethyl derivatives do not enter the brain of the rat either; the latter has been found less potent than, and the former equal in potency to compound TK 174 in enhancing the peripheral effects of noradrenaline. These derivatives do not penetrate into the young chick's brain in spite of the absence of blood-brain barrier in this animal. The N-benzyl derivative of TK 174 does not potentiate the effects of noradrenaline. β,β-Bis-(4-aminophenyl)-proprionitrile given intravenously can readily be demonstrated in the brain of rats and chicks. Compound TK 174 has been shown to exert no appreciable effect on the EEG of the chick. The failure of compound TK 174 and its immediate derivatives to enter the central nervous system is attributed to their poor lipid solubility.

The characteristic pharmacological effect of γ,γ-bis-(4-aminophenyl)-propylamine (TK 174) is the potentiation of noradrenaline (Leszkovszky *et al.* 1967a) which is a common action of several drugs such as *e.g.* cocaine and imipramine which have marked central nervous effects. It seemed therefore interesting to investigate the central nervous effects of TK 174 in an attempt to obtain information about the relationship between potentiation of noradrenaline effects on one hand, and various central effects, on the other. Previous observations have, however, shown that TK 174 has no central nervous effects, it does not even enter the brain (Leszkovszky *et al.* 1967b; Leszkovszky and Tardos 1968).

The present investigation was undertaken with the aim of finding among the derivatives of TK 174 a compound which penetrates into the brain and exerts there some effects. In addition, a series of experiments was carried out to establish whether TK 174 exerted any effect on, or could be demonstrated in the brain of such animals which normally have no blood-brain barrier.

Methods

Of compounds with similar chemical structure, the N-methyl, N-benzyl and N,N-dimethyl derivatives of TK 174, and β,β-bis-(4-aminophenyl)-propionitrile were tested. The structure of the compounds, the symbols designating them and their toxicity characteristics are seen in *Table I.*

The N-methyl derivative (SA 83) was available as a monoacetate, the N-benzyl (TK 486) and the N,N-dimethyl derivative (TK 474) as acid maleates, the nitrile compound (TK 11) as a free base, and compound TK 174 both as a free base and a monoacetate. Doses and concentrations refer to the free bases.

Toxicity was studied in acute experiments on mice and rats. The animals received the drugs intravenously, mice in 0.1 ml/10 g, and rats in 0.1 ml/100 g body weight. The number

of animals perished was established 20 min after administration of the drug, death never occurred later. The LD_{50} with its fiducial limits was calculated by the graphic method of LITCHFIELD and WILCOXON (1949).

Potentiation of the effects of noradrenaline. Comparative studies were undertaken on isolated guinea pig *vas deferens* preparations suspended in 30 ml of Locke solution aerated with oxygen at 32 °C, to an isotonic lever of 18-fold magnification under an initial tension of approximately 1 g. Contractions were elicited by the addition of noradrenaline bitartarate. Compounds to be tested were added to the medium 30 sec before the addition of noradrenaline. The effect of noradrenaline was considered potentiated if the contraction in response to it was by at least 33% larger than immediately before the addition of the substance.

Enhancement of the effects of noradrenaline was studied also *in vivo*, in spinal cat preparations in which the spinal cord had been transected under ether anaesthesia, at the level of the second cervical vertebra. The compounds to be tested and noradrenaline bitartarate (1 μg/kg) were given intravenously. The effect of the latter was considered to be enhanced if the pressor response to it was by at least 33% greater than before the administration of the compound to be tested.

Hypnotic potentiation activity. Mice were intravenously injected with 40 mg/kg of ethylbuthylthiobarbital sodium 30 min after the subcutaneous administration of the compounds to be tested and the duration of anaesthesia was estimated. The criterion of a sufficiently deep anaesthesia was the loss of the righting reflex. Potentiation of the hypnotic was considered to have occurred if the duration of anaesthesia was prolonged by at least 100% as compared to that of control animals given the same hypnotic on the same day without any pretreatment.

Inhibition of pentetrazole convulsions. Pentetrazole was intraperitoneally administered to mice, and the abolition of clonic seizures of all four extremities was regarded as the sign of anticonvulsive activity.

In some cases also the method of JENNEY and PFEIFFER (1956) based on the estimation of threshold doses of convulsants given in slow intravenous infusion was used, which is suitable for the estimation of facilitation of seizures as well as of anticonvulsive activity.

Antagonism to nicotine toxicity. Thirty min after the administration of the compounds to be tested, mice were intravenously given LD_{100} of nicotine established on the same day; this varied between 6 and 8 mg/kg of nicotine bitartarate. Survival was considered to be a sign of antagonism to nicotine. When possible, medium effective doses were calculated by the method of LITCHFIELD and WILCOXON (1949).

Determination of tissue concentrations. The compounds were injected in a volume of 0.3 ml/100 g body weight into the tail vein of rats, and into the axillary vein of chicks. At an appropriate time, the animals were decapitated in light ether anaesthesia, their organs were removed and weighed. From the larger organs 1 g specimens and smaller organs *in toto* were homogenized for 10 min in 15% trichloroacetic acid in a Potter apparatus. The homogenates were made up with 15% trichloroacetic acid to 10 ml, then filtered; 3.0 ml samples of the filtrate were transferred into test tubes. Estimation was undertaken by the colorimetric diazotation method of ÉLLŐ (1959) (for details, see LESZKOVSZKY and TARDOS 1968). Concentrations given in the *Tables* are expressed in μg/g of wet tissue (mean $\pm$ S.E.M.).

EEG studies. Electrocorticograms of chicks less than 2 weeks of age were recorded in bipolar leads. Two electrodes were implanted into the skull and fixed with dental cement above the left hemisphere under local anaesthesia induced with 1% adrenaline-free procaine. Reference electrode was implanted above the frontal sinus. The records were taken with the chicks placed in an electrostatically shielded and grounded cage. Insulated copper wires 0.3 mm in diameter were soldered to the extenuated tips of the implanted electrodes; the majority of the heat produced by soldering was conducted away through a forceps attached to the electrode. This method of protecting the animal from the generated heat is similar to that used in electrotechnics to protect transistors from heat when soldering. The wires were shielded with a common grounded grid. Records were made by an Elema-Mingograf 81 universal amplifier, with an upper frequency limit of 15 c/s, time constant of 0.6 sec, calibration of 100 μV.

Results

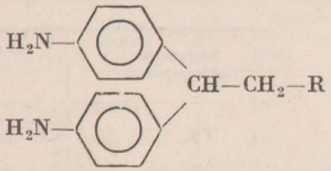
Toxicity. Results are shown in *Table I.*

The compounds displayed no particular acute toxicity. There was no substantial difference between lethal doses of the various amine derivatives; the

Table I

Chemical structure and intravenous toxicity of the compounds tested

General formula:



Designation	Formula R	Toxic dose, mg/kg intravenously			
		mouse		rat	
		LD_{50}	fiducial limits	LD_{50}	fiducial limits
TK 174	$-CH_2-NH_2$	38.2	35.7—40.9	58.0	47.1—71.4
TK 174	$-CH_2-NH_2^*$	31.2	27.6—35.6	56.0	45.9—68.4
SA 83	$-CH_2-NH_2-CH_3$	43.8	38.1—49.1	95.5	91.0—106 0
TK 486	$-CH_2-NH-CH_2-$ 	43.7	38.3—49.2	—	—
TK 474	$-CH_2-N(CH_3)_2$	43.8	38.1—50.5	—	—
TK 11	$-C\equiv N$	172	127—232	246	218—278

* acetate

toxicity of TK 174 was not influenced by the formation of the acetate. The nitrile derivative TK 11 proved in acute experiments to be 4 to 5 times less toxic than the amine compounds.

Potentiation of the effects of noradrenaline. The smallest effective concentration has been established *in vitro* for all the compounds tested *(Table II)*.

Compound TK 486 was ineffective. Enhancement was slight with TK 474 and TK 11, whereas SA 83 exerted a potentiating effect similar to that of TK 174. *Fig. 1* shows the dose-response curve for 83 SA, in comparison with the acetate of TK 174. Compound SA 83 at 1.6×10^{-7} concentration had a potentiating effect similar to that of TK 174.

Compounds TK 486 and TK 474 were tested also *in vivo*; the results agreed well with those obtained *in vitro* in that in doses up to 3 and 5 mg/kg, respectively, neither compound enhanced the pressor response to noradrenaline of the spinal cat preparation.

Hypnotic potentiation activity. In doses ranging between 25 and 100 mg/kg, none of the tested compounds prolonged the thiobarbital anaesthesia. Higher doses were not tested.

Pentetrazole seizures were not prevented by any of the compounds given subcutaneously in doses between 50 and 80 mg/kg. In the case of TK 11, an oral

Table II

Potentiation of the effect of noradrenaline in vitro

Designation of compound	Lowest effective concentration, g/ml
TK 174	4.01×10^{-8}
SA 83	4.05×10^{-8}
TK 486	$\geqslant 5.34 \times 10^{-6}$
TK 474	9.95×10^{-7}
TK 11	5.00×10^{-6}

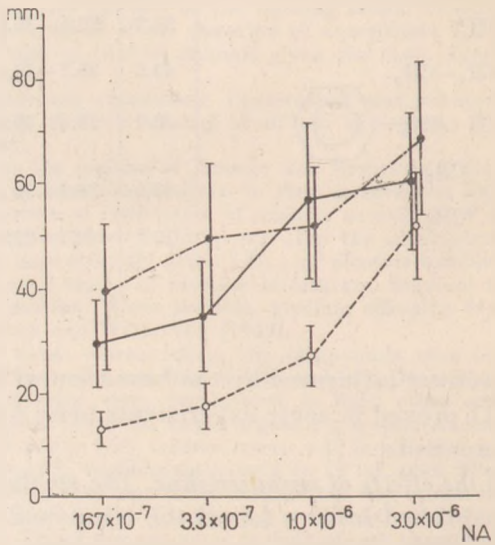


Fig. 1. Dose-response curves of noradrenaline on the isolated guinea pig vas deferens. *Abscissa*, concentration of noradrenaline bitartarate; *ordinate*, contractions as measured on the kymogram, in mm (mean + S.E.M.). ○ ····· ○ noradrenaline alone; ● —— ● noradrenaline in the presence of 1.60×10^{-7} TK 174; ● – · – · – ● noradrenaline in the presence of 1.62×10^{-7} SA 83

dose of 100 mg/kg was given. Compounds TK 174 and SA 83 in subcutaneous doses of 80.2 and 81.0 mg/kg, respectively, were also studied by the method of JENNEY and PFEIFFER (1956). The dose of pentetrazole necessary to evoke the first twitch, on the one hand, and persistent convulsions, on the other, did not differ significantly ($p > 0.10$) in the control group and in those treated with the compounds.

Antagonism to nicotine toxicity. The doses and responses are shown in *Table III*. None of the propylamine derivatives showed any antagonism to nico-

Table III

Antagonism to nicotine toxicity in the mouse

Designation of compound	Route of administration	Dose, mg/kg	Number of animals	
			Total	Survived
TK 174	subcutaneously	100.0	10	0
TK 174*	subcutaneously	80.2	10	2
SA 83	subcutaneously	81.0	10	0
TK 486	subcutaneously	53.5	10	0
TK 474	subcutaneously	49.7	10	2
TK 11	orally	$ED_{50} = 18.0$ (10.3—31.5) mg/kg		

* acetate

tine in mice. Compound TK 11 was remarkably different in this respect since in small doses it potently inhibited the toxicity of nicotine. It should be emphasized that in a previous work (Leszkovszky *et al.* 1966) this compound has been shown to possess a significant inhibitory effect on electroshock and strychnine-induced convulsions, and also to inhibit specifically polysynaptic spinal reflexes.

Compound TK 486 had neither a central effect, nor did it potentiate the effect of noradrenaline. It has therefore been discarded from further study and its tissue concentrations have not been established.

Tissue concentrations. In the rat, the compounds were determined in the brain, liver and kidney, as it has been found previously (Leszkovszky and Tardos 1968) that compound TK 174 was stored particularly in the liver and kidney. TK 174 was used in this study in the form of the free base; results are shown in *Table IV*.

It was evident from these findings that TK 174 does not enter the brain. The low concentrations observed in the brain cannot be considered significant biologically. In the liver, concentration was high and decreased only slightly with time. In the kidney, the initial concentration was relatively high, 190 μg/g, and on subsequent two measurements it showed an approximately geometric decrease.

Determination of SA 83 revealed essentially the same results as those obtained for TK 174. The concentrations found in the brain, although higher than those for TK 174, were still at the border of detectability. Five minutes after the administration of 15.8 mg/kg the concentration in the kidney was about half of that found 5 min after the injection of 31.6 mg/kg. Hepatic concentration was also lower whereas in the brain after both doses the same extremely low concentrations, 0.98 and 0.92 μg/g, respectively, were obtained. A few animals had been given 63.2 mg/kg, *i.e.* twice the usual dose. It was lethal for most of them,

Table IV

Tissue concentrations in the rat, after intravenous administration

Compound	Dose, mg/kg	Time, min	No. of. anim-als	Tissue concentration in μg/g (mean + S.E.M.)		
				Brain	Liver	Kidney
TK 174	30.0	1	10	0.58 ± 0.12	43.17 ± 3.74	195.26 ± 21.73*
		5	6	0.14 ± 0.08	45.14 ± 4.80	86.32 ± 5.56**
		15	6	0.36 ± 0.15	40.28 ± 3.97	40.38 ± 2.09**
SA 83	31.6	1	6	1.76 ± 0.13	41.83 ± 3.17	205.42 ± 14.82
		5	6	0.98 ± 0.26	51.31 ± 2.83	99.13 ± 6.50
		15	6	1.24 ± 0.25	36.93 ± 2.82	45.22 ± 5.00
	15.8	5	7	0.92 ± 0.23	34.10 ± 1.59	49.27 ± 1.74
TK 474	34.4	10	10	0.05 ± 0.05	32.19 ± 1.89	—
TK 11	30.0	1	7	26.33 ± 1.50	39.04 ± 5.91	41.26 ± 3.07
		5	6	19.20 ± 1.30	43.20 ± 3.16	28.29 ± 2.34
		10	6	14.75 ± 1.38	32.95 ± 2.79	25.35 ± 3.24

* n = 6
** n = 12

the two survivors exhibited 5 min after the administration of the compound 3.14 and 2.55 μg/g in the brain, 88.23 and 91.18 μg/g in the liver, and 300.13 and 414.97 μg/g in the kidney.

Compound TK 474, 10 min after the intravenous injection of 34.4 mg/kg, could not be detected in appreciable amounts in the brain, whereas in the liver the concentration was 32.0 μg/g, not much lower than for TK 174 and SA 83.

Distribution of compound TK 11 in the rat was entirely different. Well-detectable concentrations were found in the brain at three different intervals after the injection of the compound. The values showed a successive reduction with time, nevertheless, 15 min after the introduction the level was still 15 μg/g. In the kidney, on the other hand, there was much less of TK 11 than of the other compounds, and the rate of reduction of the values was also lower. These data are in agreement with the marked central effect of TK 11 and indicate furthermore that this compound may differ in its fate in the body from the other compounds tested.

In the chick the compounds were detected in the same tissues and organs as in the rat. The doses of TK 174, SA 83 and TK 11 were the same, and the animals were killed 1 or 10 min after the injection of the compound. Results are shown in *Table V*.

The distribution of TK 174 and SA 83 was the same in the two species, and TK 11 displayed a different distribution also in the chick. It appeared in the brain in well detectable amounts, but the level diminished more rapidly

Table V

Tissue concentrations in the chick, after intravenous administration

Compound	Dose, mg/kg	Time, min	No. of animals	Tissue concentration in μg/g (mean ± S.E.M.)		
				Brain	Liver	Kidney
TK 174	30.0	1	5	0.75 ± 0.41	56.67 ± 3.68*	174.73 ± 10.62*
		10	5	0.08 ± 0.08	46.67 ± 5.97	69.00 ± 10.78
SA 83	31.6	1	5	0.86 ± 0.31	49.80 ± 7.86	117.81 ± 10.59
		10	5	0.78 ± 0.22	41.47 ± 4.03	60.06 ± 4.94
TK 11	30.0	1	5	24.61 ± 3.35	52.69 ± 3.12	47.30 ± 3.58
		10	5	3.99 ± 0.47	31.51 ± 2.56	22.80 ± 4.41

* n = 7

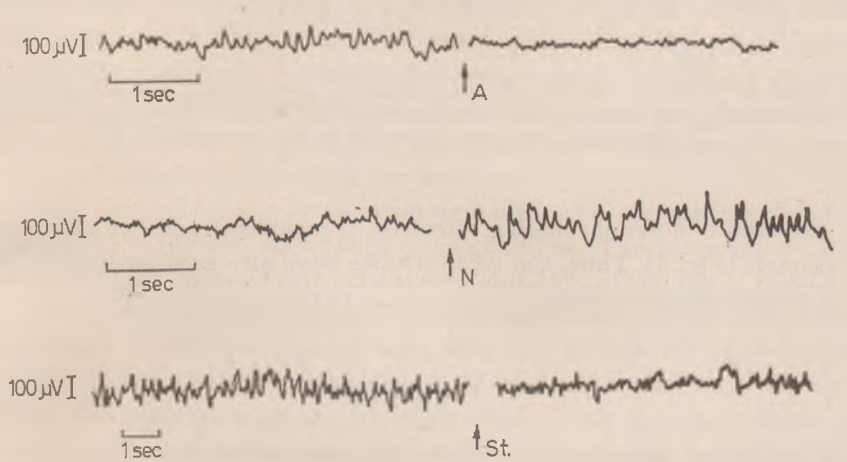


Fig. 2. Effect of amphetamine, noradrenaline and external stimuli on the EEG of young chicks. Upper tracing, 9 days old chick weighing 70 g, A = amphetamine phosphate, 7 mg/kg intravenously; recording continued 20 sec after the injection. Middle tracing, 13 days old chick weighing 81 g, N = noradrenaline bitartarate, 30 μg/kg intravenously; recording continued 20 sec after the injection. Bottom tracing, 13 days old chick, weighing 93 g, St = sensory stimulation

than in the rat brain, although at 1 min the values were practically the same. As in the rat, the renal concentration of TK 11 was lower than that of compound TK 174.

EEG studies. Similarly as in other species, in the chick the resting state and sleep are characterized by high-amplitude low-frequency EEG-activity; alertness, excitation, external stimuli cause desynchronization. Adrenaline and noradrenaline give rise to a sleep-like state which is characterized by synchronization, whereas amphetamine, similarly as in other species, evokes alertness and desynchronization (Spooner and Winters 1965; Key and Marley 1962).

In the present experiments, these well known EEG effects of noradrenaline and amphetamine could be reproduced. Both agents were given intravenously, in doses of 30 μg/kg and 7 mg/kg, respectively. In addition, the desynchronizing effect of external stimuli could also be demonstrated *(Fig. 2)*.

Compound TK 174 in an intravenous dose of 30 mg/kg had no appreciable effect on the EEG of chicks: neither caused it desynchronization in the resting

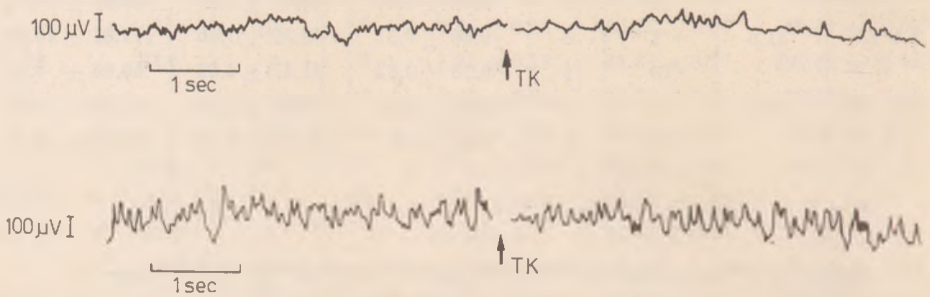


Fig. 3. Effect of TK 174 on the EEG of young chicks. Upper tracing, 9 days old chick weighing 64 g; middle tracing, 13 days old chick weighing 81 g; TK = TK 174, 30 mg/kg intravenously. Recording continued 20 sec after the injection

state, nor had it a synchronizing effect on the cortical activity of previously alert animals (Fig. 3). Thus, the EEG studies have also confirmed that TK 174 has no central nervous effect.

Discussion

Clarification of the relationship between noradrenaline potentiation and central nervous effects would probably throw light on the mechanism of action of thymoleptics such as imipramine and its derivatives (Haefeley *et al.* 1964). Glowinski and Axelrod (1964) revealed a marked relationship between the antidepressive effect and the inhibition of noradrenaline uptake by cerebral neurones. In a previous study it was concluded that the enhancement of noradrenaline effects by TK 174 was similarly based on the inhibition of noradrenaline uptake (Leszkovszky *et al.* 1967b). It seemed therefore interesting to investigate the central nervous effects of the compound; earlier (Leszkovszky and Tardos 1968) and the present results indicated, however, that TK 174 does not enter the brain.

In view of the above, the effect of compounds with related chemical structure was investigated. For this purpose, the aliphatic amino group was converted into a secondary or tertiary amino radical. The greatest expectation was attached to SA 83, simply because tricyclic antidepressants also have dimethylamino or methylamino groups at the end of the aliphatic carbon chain while in

none of the active agents of this type is there a primary amino radical at the end of the side chain. Of the corresponding secondary and tertiary amine derivatives it is usually the secondary one which has a stronger biological effect. Desmethylimipramine, for example, is the active metabolite of imipramine, although it displays also some qualitative differences from imipramine (MESNARD and DEVAUX 1967; THEOBALD *et al.* 1964; GIURGEA and DAUBY 1965; KAUMANN *et al.* 1964; HOROVITZ *et al.* 1964; KIELHOLZ and PÖLDINGER 1968). This is why we hoped that, by converting the alphatic primary amino group of TK 174 into a tertiary radical, and particularly into N-methyl secondary amine, a compound readily entering the central nervous system could be obtained.

The present results indicate that although the N-methyl secondary amine derivative (SA 83) has a more pronounced potentiating effect on noradrenaline than the N,N-dimethyl tertiary amine derivative (TK 474), its effect is not superior to that of the primary amine (TK 174), and none of them enters in appreciable amounts the central nervous system. The N-benzyl derivative (TK 486) is also a secondary amine; this, however, was completely ineffective. It seems that the substituting radical which ends in a benzene ring alters the size of the molecule too much. As the compound proved to have no biological effect, tissue concentrations were not measured.

Tissue concentrations were estimated after the administration of large doses in order to obtain detectable concentrations. The intravenous dose of TK 174 and 83 SA given to rats attained 49 and 34% of the LD_{50}, respectively. Even these doses caused collapse and transient respiratory disturbances in some of the animals. It may be supposed that they damaged the blood-brain barrier and this could have accounted for the small amounts found in the brain. This possibility was challenged by the fact that SA 83 in half the above doses, which did not give rise to any symptom, appeared in the same very low concentration (0.98 and 0.92 μg/g) in the brain after 5 min. Even after the administration of twice the above dose, which was markedly toxic, the SA 83 level varied between 2 and 3 μg/kg, values consistently lower than those found for TK 11 when the latter was given in smaller doses. The observed low levels may therefore be due to contamination with extracerebral tissue, or even to methodical errors.

Since in acute experiments TK 11 proved to have a low toxicity, 30 mg/kg was given intravenously; this corresponded to 12% of the LD_{50}. In the brain, the compound appeared in well detectable concentrations. The positive result obtained with this substance which has a marked central effect has supported the reliability of the negative findings obtained with the other compounds.

It is known from the literature that chicks until the fourth to fifth week of life do not have a blood-brain barrier, thus, pharmacologically active agents such as noradrenaline and 5-hydroxy-tryptamine, which otherwise do not penetrate the blood-brain barrier can be revealed in the brain of the young chick (ALLEN and MARLEY 1961; KEY and MARLEY 1962; KOBRIN and SEIFTER 1966;

RAUZZINO and SEIFTER 1967; SPOONER and WINTERS 1965). This is why we used chicks in the present experiments assuming that compound TK 174 which did not penetrate the barrier in the rat would appear in the brain of the chick. This hypothesis, however, was not supported by our results.

In the EEG the well-known effects of noradrenaline and amphetamine could be reproduced, but compound TK 174 had no appreciable effect. This observation together with the other results is a proof of TK 174 having no central nervous effect.

The finding that the compound does not enter the brain in the chick indicates that it is not the blood-brain barrier which prevents the agent from reaching the central nervous system. Presumably, the compound's lipid solubility is poor and therefore it cannot attain sufficiently high concentrations in the central nervous system. Compound TK 11, on the other hand, which contains a nitrile instead of the aliphatic amino group, has been shown readily to enter the brain and to have marked central nervous effects. It is, however, less effective in potentiating the effect of noradrenaline.

By attaching a methyl group to the aliphatic amino radical of TK 174 a compound was obtained which markedly augmented the effects of noradrenaline. This alteration in the structure, however, was insufficient to attain a reduction of the hydrophilic property of the molecule which was obviously responsible for the failure of the compound to exert a central nervous effect.

*

The valuable technical assistance of MR. I. SAJÓ is gratefully acknowledged.

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EFFECT OF A FLAVONOID COMPOUND ON THE K TRANSPORT OF ERYTHROCYTES

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(Received July 9, 1968)

In Wilbrandt's "depolarized erythrocytes" the effect on K transport of a 7-oxyflavone-dimethylamino derivative has been studied. At 37 °C the compound at 0.2 mM concentration significantly reduced K loss by the erythrocytes. Decamethonium at 1 mM concentration showed a similar effect, while 5 mM of physostigmine sulphate practically abolished K outflow. In the presence of Ca ions (which likewise act as inhibitors) K loss was inhibited by the flavonoid completely even at 0.1 mM concentration. At 2 °C no K loss occurred thus the process is temperature sensitive.

In the past decades evidence has been accumulating as to the biological effects of flavonoids. The most detailed survey of the results obtained has been published by Böhm (1959, 1960). In our own investigations we have undertaken to study the flavonoid effect at the molecular level. It was found that a 7-oxyflavone preparation inhibited the contractions of the frog sartorius induced by KCl or caffeine and profoundly influenced the changes in membrane polarization brought about by KCl (Heténvi 1968a). In the present work the effect of the flavonoid compound on K transport was studied by means of an erythrocyte model.

Methods

In the experiments Wilbrandt's "depolarized erythrocytes" were used. The method of Lundsgaard-Hansen (1957) was slightly modified as follows. Human blood obtained from a vein was defibrinated with a wooden stick, filtered through gauze, the plasma was centrifuged, and the residue was washed three times with isotonic sucrose solution containing 10% isotonic NaCl. The washed erythrocytes were suspended in a solution composed of 3% isotonic NaCl and 97% of isotonic sucrose, to make a 2% suspension, as controlled by haematocrit. The suspension was incubated at 37 °C. Samples taken at intervals were immediately cooled, centrifuged, the supernatant was removed by suction, the sediment was made up with distilled water to the desired volume and haemolyzed, and the K in the haemolysate was determined by flame photometry.

Several flavonoid compounds were tested for their effect on the K loss by depolarized erythrocytes. A 7-oxyflavone-dimethylamino derivative ($C_{19}H_{19}O_3N \cdot HCl$, Ms: 346, in the following: S_{12}) showed the highest activity. In other experiments the effect of 5.0 mM physostigmine sulphate and 1.0 mM decamethonium were studied in a similar fashion. S_{12} was added to the incubation mixture at a concentration of 0.2 mM. These concentrations were selected to facilitate comparison with our previous muscle experiments.

In the experiments in which the effect of Ca ions on K loss was studied, sufficient isotonic $CaCl_2$ was added to the incubation mixture to obtain a total electrolyte content identical with that of the control which was 3% (1.8% isotonic NaCl + 1.2% isotonic $CaCl_2$ + 97% isotonic sucrose).

Results

The control experiments have confirmed the finding by LUNDSGAARD-HANSEN (1957) in that by the end of the 40 minute observation period a con-

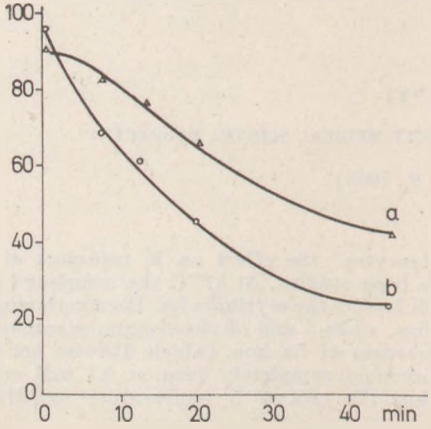

Fig. 1. Inhibitory effect of 7-oxyflavone derivative on K loss from depolarized erythrocytes. Ordinate: K content of erythrocytes in mEq/l; abscissa: time in min. *a:* 0.2 mM flavone. *b:* control

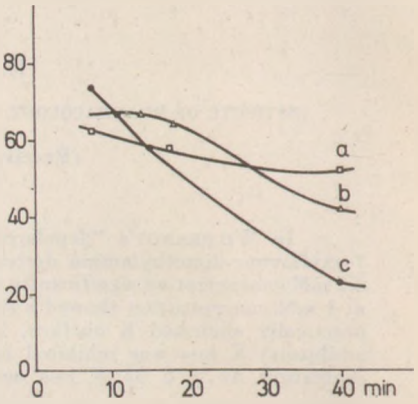

Fig. 2. Inhibition of K loss from erythrocytes by physostigmine sulphate and decamethonium. Ordinate: K content of erythrocytes in mEq/l, abscissa: time in min. *a:* 5.0 mM physostigmine. *b:* 1.0 mM decamethonium. *c:* control

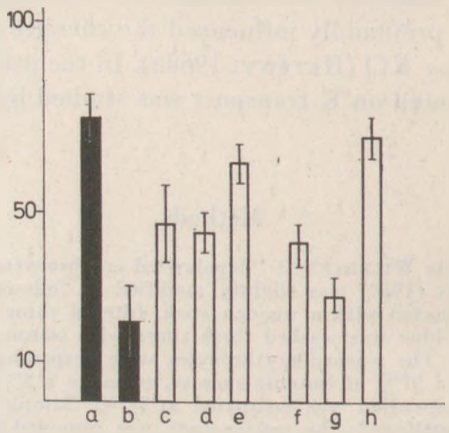

Fig. 3. Inhibition of K loss from depolarized erythrocytes by 7-oxyflavone, decamethonium and physostigmine, as well as by Ca ions and Ca ions + flavone. Mean K content (mEq/l) of erythrocytes *a:* at onset of incubation (n = 15), *b:* after 40 minutes incubation (control; n = 15), *c:* in the presence of 0.2 mM flavone, *d:* 1.0 mM decamethonium, *e:* 5.0 mM physostigmine, *f:* Ca ions, *g:* 0.1 mM flavone and *h:* Ca+0.1 mM flavone, after incubation

siderable quantity of K passes from the depolarized erythrocytes into the given medium. On the other hand, in 15 experiments 0.2 mM S_{12} definitely diminished the K loss; the difference from the controls was significant *(Fig. 1).*

Since physostigmine and decamethonium inhibit Na transport in both directions in the frog's sartorius muscle (KOVÁCS *et al.* 1966; SZABÓ *et al.* 1966), we studied their effect on K loss in the erythrocyte model. In 10 experiments each, both compounds proved to inhibit K loss. The effect of 1.0 mM decamethonium was about the same as that of 0.2 mM S_{12}, while 5 mM physostigmine almost completely inhibited K outflow (*Figs 2* and *3a—e*).

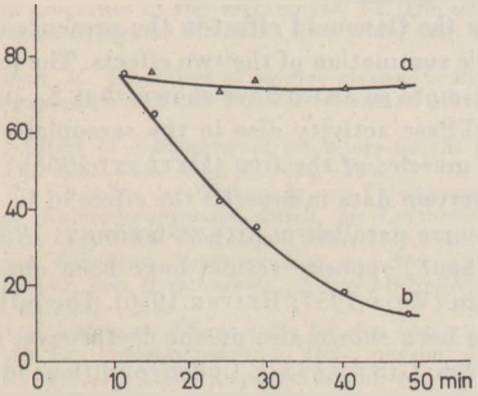

Fig. 4. Effect of temperature on K loss by depolarized erythrocytes. *a:* 2 °C, *b:* 37 °C

These observations have not only confirmed the results obtained by LUNDSGAARD-HANSEN in that Ca ions diminished the K loss of depolarized erythrocytes, but also showed that in the presence of Ca ions S_{12} at the otherwise hardly active concentration of 0.1 mM completely inhibited the K loss. The results of 10 experiments each of this kind are shown in *Fig. 3f, g* and *h*.

K outflow from depolarized erythrocytes is considered to be a passive process. On the other hand, in our experiments while at 37 °C there occurred the usual K loss, in experiments carried out under the same conditions but at 2 °C practically no K was released during the 50 minute observation period *(Fig. 4)*.

Discussion

WILBRANDT's depolarized erythrocytes seemed to be suitable not only for studying ion movements, but the results obtained have well supplemented our results obtained on muscle preparations. Without over-generalization, the K loss by erythrocytes in the above medium may to some extent be considered parallel with the depolarization of muscle cells during stimulation. It has been shown that during incubation in sucrose solution the erythrocytes suffer an osmotic loss (JACOBS 1932, 1933) and that this is based on an escape of intracellular K (DAVSON 1939). WILBRANDT (1960) reported that on incubation in

isotonic NaCl+isotonic sucrose, the medium used also by us, the erythrocyte membrane shows depolarization, the measure of which is determined by the ratio of intra- and extracellular Cl (LUNDSGAARD-HANSEN 1957).

Thus, K outflow from the depolarized erythrocytes may be considered a model of the ion shifts taking place in excited muscle cells. The present results have thus confirmed from another angle the depolarization-inhibiting effect of S_{12} demonstrated in frog muscle.

The increase in the flavonoid effect in the presence of Ca ions cannot be explained by a simple summation of the two effects. The problem thus requires further analysis, the more so as we have shown that S_{12} profoundly influences Ca transport and ATPase activity also in the sarcoplasmic reticular fraction prepared from limb muscles of the frog (HETÉNYI 1968b).

According to certain data in muscles the effect of Ca ions and digitalis on K transport shows some parallelism (SZENT-GYÖRGYI 1956).

On the other hand, opposite results have been obtained concerning Ca ions and strophanthin (WITT 1957; REITER 1956). The influence of digitalis on the cation pump has been shown also on the erythrocyte membrane (SCHATZMANN 1953; KAHN 1955; GLYNN 1957). Under conditions identical to those used by us, experiments with depolarized erythrocytes showed, however, that the outflow of K was inhibited only by the Ca ions and not by strophanthin (LUNDSGAARD-HANSEN 1957). In the light of these data our experiments seem to indicate that the site of action of S_{12} in depolarized erythrocytes differs from that of cardiac glycosides as far as K transport is concerned. Our isotope studies to elucidate this are in progress. — We intend to use our results obtained with decamethonium and physostigmine for comparison with the muscle experiments. The effect proved to be the same in the erythrocyte model, emphasizing again the manifold similarity of the two tests.

The K loss by depolarized erythrocytes, as an ion movement in the direction of the concentration gradient, is generally believed to be a passive process. Although diffusion is also sensitive to temperature, according to our observations this movement as also the depolarization of muscle cell membrane in the excitatory phase, has a temperature sensitivity indicative of an active — enzymatic — mechanism. This was supported in our experiments by the calculated Q_{10} value of 2.3 to 2.5, which corresponded to the Q_{10} in enzymatic reactions.

The present results not only proved the influence on ion transport of the compound S_{12} but also yielded evidence as to the site of action.

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Recensiones

J. S. Garrow, R. Smith and E. E. Ward

Electrolyte Metabolism in Severe Infantile Malnutrition

Pergamon Press, Oxford, 1968. Price 67/6 s.

This interesting monograph of 168 pages is divided into 11 chapters and contains numerous excellent references.

The authors are recognized investigators of infantile malnutrition, especially of its salt-water metabolism. The book deals with the results of the past 20 years' investigations into the problems of malnutrition beginning with the author's own research work in *Jamaica*. Due mention is made of more important experiments in other parts of the world, thus also of those carried out in *Hungary*. Malnutrition is defined, its clinical aspects and its relation to gastro-enteritis are described. Changes in body composition and in sodium, potassium and magnesium metabolism are treated in great detail. Two special chapters are devoted to renal and hormonal functions, on the one hand, and to mortality, prognosis, and therapy of malnutrition on the other.

The illustrations are clear. The book will be useful for paediatricians and nutritionists, and in postgraduate teaching.

F. Varga

F. Ghiretti (Ed.)

Physiology and Biochemistry of Haemocyanins

Academic Press, London and New York, 1968.
Pp ix + 133. Price 40 s.

The volume contains 10 papers presented at an International Symposium held at the Zoological Station of Naples in 1966. Although the emphasis was laid on haemocyanins, papers are included on coeruloplasmin and haemerythrin. Most of the papers are short reviews, defining particular problems of the structure and function of haemocyanins. The most interesting part of the book seems to be the one dealing with the state of copper in different biological systems. The volume would be more useful had there been included a synoptic paper.

In spite of the book being obviously designed for biochemists, it will be useful for all scientists concerned with the problems of comparative biochemistry and physiology.

G. Dénes

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BUENOS AIRES, REPUBLICA ARGENTINA

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РЕЗЮМЕ

ДЕЙСТВИЕ ИЗМЕНЕНИЙ КРОВЯНОГО ДАВЛЕНИЯ НА АКТИВНОСТЬ КЛЕТОК БУЛЬБАРНОГО СЕТЧАТОГО ОБРАЗОВАНИЯ

И. ПОРСАС и К. ГИБИСЕР-ПОРСАС

Действие изменения кровяного давления на активность клеток бульбарного сетчатого образования кошек было исследовано с помощью микроэлектродов. Рефлексы со стороны кровообращения были выключены путем билатерального пересечения блуждающего нерва, а также нервов синуса и гломуса сонной артерии. Понижение кровяного давления, вызываемое ацетилхолином и раздражением центральной и периферической культей блуждающего нерва, в общем оказывает повышающее действие на активность клеток, тогда как повышение кровяного давления вызывает торможение самых различных активностей клеток. На основе экспериментальных результатов авторы придерживаются того мнения, что ацетилхолин не вызывает специфической активации активности клеток и что повышение последней после понижения кровяного давления обуславливается местной асфиксией. Для объяснения тормозящего действия повышения кровяного давления авторы выдвигают предположение, что клетки или непосредственно чувствительны к повышению давления, или в кровообращении мозга присутствуют прессорецепторы, которые гистологическими методами исследования еще не удалось выявить.

ПРОБЛЕМА СООТНОШЕНИЯ МЕЖДУ ПОРОГОМ РАЗДРАЖЕНИЯ И ОПЕРАТИВНЫМИ УРОВНЯМИ В РЕАКЦИЯХ ЧЕЛОВЕКА НА РАЗЛИЧНЫЕ АКУСТИЧЕСКИЕ РАЗДРАЖЕНИЯ

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Методом комплексной регистрации реакций ЭМГ, ЭЭГ и ГБР показывается связь между порогом анализатора и различными оперативными уровнями. При последовательном анализе данных представилась возможность для следующих выводов:

1. Скрытый период реакции ЭМГ зависит от близости внешнего актуального раздражения к интенсивности сохранившегося в памяти стандартного раздражения, служащего для сравнения. В то же время последующее действие ЭМГ определяется исключительно лишь интенсивностью раздражения.

2. Скрытый период и последующее действие ЭЭГ и ГБР предположительно определяются различным периодом суммирования нейрональных элементов «on» и «off».

3. Действие, регулирующее появление ответов ЭЭГ и ГБР в качестве компонентов реакции ориентации зависит, с одной стороны, от физикальной силы раздражения, а с другой, от процессов, осуществляющих сравнение актуального раздражения с сохранившемся в памяти стандартным раздражением.

4. Механизм ошибочных реакций возбуждения предположительно следует искать не только в «собственном шуме» анализатора, он тесно связан также с флюктуацией чувствительности оперативных уровней.

5. Появление реакций ЭЭГ и ГБР независимо от сохранившегося в памяти стандартного раздражения указывает на то, что анализатор, кроме различных оперативных уровней, служащих для осуществления двигательных реакций, обладает также постоянным порогом раздражения.

РОЛЬ ГИПОФИЗА В РЕГУЛИРОВАНИИ СОДЕРЖАНИЯ ГЛИКОПРОТЕИДОВ В СЫВОРОТКЕ

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В процессе развития скипидарового абсцесса повышаются все исследовавшиеся углеводные фракции гликопротеидов. После введения вытяжки гипофиза повышение более значительное. Подобные изменения показывает также количество углеводных фракций гликопротеидов в печени. Тот факт, что реакция гликопротеидов наблюдается также после экстирпации гипофиза, указывает на то, что эта реакция управляется филогенетически более древней регуляционной системой, по всей вероятности посредством индукции синтеза белков. С другой стороны, наблюдение, что гипофиз влияет на степень реакции, позволяет заключить, что в высших организмах филогенетически более древний регуляционный механизм подчинен гормональной регуляции, управляемой гипофизом.

ДЕЙСТВИЕ ДЕГИДРАТАЦИИ И ПОВТОРНОЙ ГИДРАТАЦИИ НА СУПРАОПТИКО-НЕЙРОГИПОФИЗАРНУЮ НЕЙРОСЕКРЕТОРНУЮ СИСТЕМУ И НА СОДЕРЖАНИЕ АДГ В НЕЙРОГИПОФИЗЕ КРЫС

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Авторы исследовали действие жажды и повторной пробы с нагрузкой водой на активность АДГ нейрогипофиза крыс и на содержание нейросекрета в nucleus supraoptic us и нейрогипофизе (при окрашивании альдегид-фуксином).

В норме повторная проба с нагрузкой водой обуславливает повышение содержания АДГ в задней доли гипофиза. У этих животных гистологическая картина nucleus supraopticus была гипофункционального типа. После 10-дневной жажды у животных была выявлена противоположная картина. Повышение числа «мелких темных клеток» в связи с жаждой выдвигает возможность, что между этими клетками и большими клетками существует и функциональная разница. Результаты опытов показывают, что проба с нагрузкой водой уменьшает секрецию АДГ из нейрогипофиза.

ДЕЙСТВИЕ НАГРУЗКИ ГИПЕРТОНИЧЕСКИМ РАСТВОРОМ МАННИТА ПРИ ДЕГИДРАТАЦИИ ВСЛЕДСТВИЕ ЖАЖДЫ

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Четырехдневным лишением собак воды и внутривенным введением 10 мл 20%-ого раствора маннита на кг веса тела в день при неограниченном приеме пищи была вызвана экспериментальная дегидратация.

При сравнении экспериментальных результатов с изменениями, полученными при дегидратации вследствие простого лишения животных воды было установлено, что вливание гипертонического раствора маннита 1. ускоряет возникновение дегидратации, 2. повышает даже при тяжелой дегидратации выделение воды и натрия, выраженное в %-ах клубочковой фильтрации, 3. купирует канальцевую ответную реакцию, характерную для дегидратации и 4. ускоряет развитие уменьшения клубочковой фильтрации.

Экспериментальные данные доказывают, что при дегидратации от лишения животных воды в ответной реакции почек можно выделить два хорошо обособляемых (канальцевый и клубочковый) компонента. Нагрузкой раствором маннита можно выключить канальцевый компонент, причем тяжелые симптомы, типичные для дегидратации, проявляются быстрее.

ЭЛЕКТРОЭНЦЕФАЛОГРАФИЧЕСКОЕ ИССЛЕДОВАНИЕ ПРИВЫЧКИ У ЛЮДЕЙ

Э. ЭНДРЁЦИ, Т. ФЕКЕТЕ, К. ЛИШШАК и К. ОШВАТ

Установлено, что под влиянием многократного применения звукового или светового раздражения электрическая активность новой коры (неокортекса) после 50—200 повторений реагирует синхронизацией, исходящей из височной и теменной областей. Если одновременно с применением сигнала подопытное лицо производит также двигательную реакцию (сжатие рук), то впоследствии синхронизация наблюдается уже во время осуществления двигательной реакции, а часто даже предшествует последней. Актуальная психическая деятельность, заучение наизусть определенных задач не мешает синхронизации, вызванной применением тест-раздражений. Обсуждается также механизм синхронизации и ее физиологическое значение.

РАСПРЕДЕЛЕНИЕ ХЛОРОКВИНА В ОРГАНИЗМЕ КРЫСЫ ПРИ ВВЕДЕНИИ РАЗЛИЧНЫХ ДОЗ

Ф. ВАРГА

После однократной дачи хлороквина (20, 40, 80 и 400 мг на кг веса, перорально) была определена концентрация хлороквина в плазме, печени, селезенке, легких, почках, сердце, мышцах, мозге и яичках. При повышении дозы уровень хлороквина повышается в печени, сердце и в мышцах гораздо быстрее, чем в прочих органах, причем все более большой процент введенного количества задерживается в организме. После повторной дачи хлороквина (20, 40, 80 и 160 мг на кг веса в день на протяжении недели) были получены подобные результаты. При прекращении дачи повышенных доз (160 мг на кг в день) концентрация хлороквина понижается в печени, сердце и в мышцах быстрее, чем в прочих органах. После отмены меньших доз (20, 40 мг на кг в день) этого не наблюдалось. Следовательно соотношение концентрации хлороквина в отдельных органах может измениться в зависимости от того, получают ли животные низкое (нетоксическое) или высокое (уже сублетальное) количество хлороквина. Обсуждается предположительная причина этого явления.

ВНУТРИКЛЕТОЧНАЯ ЛОКАЛИЗАЦИЯ ХЛОРОКВИНА В ПЕЧЕНИ И ПОЧКАХ КРЫС

Ф. ВАРГА

В 24-й час после дачи крысам различных доз хлороквина (13—360 мг на кг веса, перорально) было определено внутриклеточное распределение хлороквина в печени и в почках. После введения 13 мг на кг веса специфическое связывание хлороквина (мкг хлороквина на мг азота) в отдельных фракциях клеток печени показывает следующие величины: гомогенизат — 0,79, ядра — 0,60, митохондрии — 2,34, микросомы — 0,34, растворимая фракция — 0,20. После дачи 360 мг на кг веса же: гомогенизат — 57,1, ядра — 38,8, митохондрии — 12,5, микросомы — 22,2 и растворимая фракция — 112. Внутриклеточное распределение хлороквина в почках также зависит от применяемой дозы (от тканевой концентрации). Между содержанием нуклеиновой кислоты и хлороквина в отдельных клеточных фракциях не наблюдается связи. Обсуждается предположительная причина зависимости внутриклеточной локализации хлороквина от применяемой дозы.

О ДЕЙСТВИИ ПРОИЗВОДНЫХ БИС-(4-АМИНОФЕНИЛ)-АЛКИЛА НА ЦЕНТРАЛЬНУЮ НЕРВНУЮ СИСТЕМУ

Д. ЛЕСКОВСКИ, Л. ТАРДОШ, Д. ШЕБЕШТЬЕН и К. ТАКАЧ

γ,γ-Бис-(4-аминофенил)-пропиламин (174. ТК.) у крысы не проникает в центральную нервную систему и не оказывает центрального действия. N-метил- и N, N-диметил-производные 174. ТК. также не проникают в мозг крысы. Последнее производное повышает периферическое действие норадреналина меньше, чем 174. ТК., а первое в такой же мере как 174. ТК. У молодых цыплят, у которых между кровью и мозгом не имеется барьера, эти производные также не попадают в мозг. N-бензоловое производное 174. ТК. не оказывает потенцирующего действия на эффект норадреналина. При внутривенном введении *β,β*-бис(4-аминофенил)-пропионитрил хорошо обнаруживается как в мозге крысы, так и в мозге цыплят. 174. ТК. не оказывает оцениваемого действия ни на ЭЭГ цыплят. Тот факт, что 174. ТК. и его прямые производные неспособны проникать в центральную нервную систему, авторы объясняют плохой липоидорастворимостью молекулы.

ДЕЙСТВИЕ ФЛАВОНОИДНОГО СОЕДИНЕНИЯ НА ПЕРЕХОД КАЛИЯ У ЭРИТРОЦИТОВ

Э. ХЕТЕНЬИ

Автор на так наз. «деполяризованных эритроцитах» Вильбрандта изучал действие флавоноидного соединения (производное 7-оксифлавон-диметиламина) на переход калия. Было установлено, что при температуре в 37° С вышеуказанный препарат в концентрации 0,2 мМ, по сравнению с контролем, достоверно понизило потерю калия у эритроцитов. Подобное действие показал декаметоний в концентрации 1,0 мМ, в то время как 5,0 мМ-овый сульфат физостигмина практически прекратил выход калия. В присутствии ионов кальция (являющихся также ингибиторами перехода калия) вышеуказанный флавоноид уже в меньшей, сама по себе еще очень мало эффективной концентрации (0,1 мМ), полностью прекратил выход калия у деполяризованных эритроцитов. При температуре 2° С потеря калия не наблюдается, следовательно этот процесс чувствителен в отношении температуры.

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