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NETHERLANDS CENTRAL INSTITUTE FOR BRAIN RESEARCH AMSTERDAM

PROGRESS REPORT 1972

History and function of the institute

In 1905, the Royal Netherlands Academy of Sciences and Letters applied to the Dutch Government for permission to found an institute for brain research. The government consented and on June 8th, 1909, the Netherlands Central Institute for Brain Research was opened. The institute was seated in a wing of the then newly erected Department of Anatomy and Embryology of the Municipal University of Amsterdam, built under the direction of L. Bolk, professor of anatomy and embryology, who, together with the neurologist professor C. Winkler, was one of the most important supporters of the institute. The first director was Dr. C. U. Ariëns Kappers who became world-famous as a comparative neuroanatomist. After some years he was appointed professor of neuroanatomy at the University of Amsterdam. After his death, in 1946, Kappers was succeeded by Professor B. Brouwer, who previously had held the chair of neurology at the University of Amsterdam. Brouwer was primarily interested in neuropathology. During his management Dr. J. Drooglever Fortuyn, at present professor of neurology at the University of Groningen, introduced electrophysiology at the institute.

After the untimely death of Brouwer, in 1949, the Dutch government agreed that the institute should be reorganized and extended. Thus it became possible to found some new divisions enabling the institute to perform multidisciplinary research in the broad field of neurosciences, which was in accordance with its original aim.

In 1952, professor S. T. Bok, who previously had held the chair of histology at the University of Leiden, was appointed director. His merit has been to further multidisciplinary research at the institute on a large scale. He was one of the first researchers in the quantitative analysis of the brain, especially of the cerebral cortex, who earned great fame. After his retirement he was succeeded by J. Ariëns Kappers, previous professor of anatomy at the University of Groningen, who at the same time was appointed professor of neuroanatomy at the University of Amsterdam.

In former times comparative neuroanatomy was the only field of research at the institute. The scientific and the technical staffs were very restricted in number. As mentioned above professor Bok changed this situation. Since then the number of staff members and of research projects has increased progressively. At the end of 1972, the total number of staff members was 80, 39 of whom were either full-time or part-time research workers.

In 1964, the institute moved to a provisional, but much larger building and some additional barracks have since been built. Plans are now ready for a final large Institute for Brain Research on the site of the new Medical Centre of the University of Amsterdam.

The institute is a governmental institution, as its financial support is exclusively supplied by the government. It is managed by a director under the supervision of a board of professors of various disciplines at Dutch universities. Members of the board are appointed by the Royal Netherlands Academy of Arts and Sciences.

At present, there are 4 main research divisions at the institute: morphology, physiology, neurochemistry and neuropharmacology. The divisions of morphology and physiology comprise several working teams such as the teams for histology and neuroendocrinology, comparative and quantitative neuroanatomy, histo- and cytochemistry, electron microscopy, experimental physiology, comparative and developmental neurophysiology, physiology of behaviour, and system analysis. The institute

has also a number of workshops and is provided with excellent technical equipment including some computers. A close contact between the members of the various divisions and special teams contributes considerably to the integration of neurobiological research. During the past years relations have been established between some teams and clinicians of the University.

Although the institute has no special funds available to enable guestworkers to work at the institute, many non-Dutch residents could do research work during the last year, subsidized by special funds or fellowships such as offered by the International Brain Research Organization. Moreover, the institute offers an opportunity to Dutch students in medicine, psychology, biology, etc. of all Dutch universities to do work on special subjects connected with their study.

Scientific Staff

J. Ariëns Kappers, Director

Division of Physiology

Section of Experimental Neurology
Head: J. P. Schadé
J. C. de Valois
W. van Emde Boas
B. M. F. v. Cranenburgh
J. P. C. Peperkamp
C. V. de Blécourt
J. P. Muizelaar
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Section of Histology and Cytology
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R. A. C. de Vries
A. R. Smith

Section of Histochemistry
Head: J. F. Jongkind
D. F. Swaab
G. J. Boer

Section of Comparative and Quantitative Neuroanatomy
Head: G. J. Smit
K. C. Hodde
P. Kenemans
C. J. van der Sloot
E. van der Wiele
H. Uylings

Section of Electron Microscopy
H. R. Romijn
J. Varela

DIVISION OF NEUROBIOCHEMISTRY

Head: B. Oestreicher
C. van Leeuwen

DIVISION OF NEUROPHARMACOLOGY

Head: J. J. Meisch
M. van Wijk

Division of Morphology

1. SECTION OF HISTOLOGY AND CYTOLOGY

Prof. Dr. J. Ariëns Kappers:

Assistance was given to H. Romijn, A. R. Smith and R. A. C. de Vries in their investigations on the rabbit and rat pineal gland (see below).

Together with H. Hodde, an investigation was started into the site(s) of termination of the nerve fibres of the pineal organ and of the parietal eye in the lizard, *Lacerta viridis*, using the method of nerve fibre degeneration. These sites are still unknown. Their determination will contribute to the understanding of the exact function of both, the pineal organ, which, in *Lacerta*, shows some photoreceptor cells and nerve cells, and the parietal eye.

Dr. A. R. Smith:

The investigations reported in the Progress Report 1971 were finished and the results published in a thesis and three other papers. In addition, a start was made with a study of the function of the epiphyseo-hypothalamo-hypophyseal-gonadal axis under different experimental conditions, such as gonadectomy, pinealectomy, drug administration and hormone implantation. Some of the results obtained are now in press. It appeared

that in both, the pineal gland and the parvocellular hypothalamic nuclei, besides 5-HT, a yellow autofluorescent substance is present. After gonadectomy and after administration of p-chlorophenylalanine, which blocks the synthesis of 5-HT, the number of autofluorescing cells increases in the pineal gland as well as in the parvocellular nuclei. An increase in number of the yellow autofluorescent cells in the parvocellular nuclei was also observed after pinealectomy. The preliminary results obtained support the hypothesis that the antigonadotropic activity, exerted by the pineal gland on the gonads, is probably not effected directly, but via the parvocellular hypothalamic nuclei.

Dr. R. A. C. de Vries:

Last year's investigations on the functional relationship of the rat pineal gland to the hypothalamic magnocellular neurosecretory nuclei under different experimental conditions (see Progress Report 1971) led to the publication of a thesis.

In april, Dr. de Vries left the Institute for a one-year visit to the Department of Physiology, director Prof. McCann, of the University of Texas at Dallas, U.S.A., to learn the radio-immuno-assay technique, especially as the gonadotropic hormones are concerned. This visit, which will be of great importance for solving some of the problems involved in the understanding of the function, exerted by the epiphysis cerebri on the hypothalamo-hypophyseal-gonadal axis, is subsidized by an IBRO/Unesco grant.

2. SECTION OF COMPARATIVE AND QUANTITATIVE NEUROANATOMY

Dr. G. J. Smit and H. B. M. Uylings:

In 1972 investigations on the branching pattern of the basal dendrites of pyramidal neurones were continued.

On the basis of measurements in a cartesian-coordinate system, a model has been formulated with respect to the topological and metrical aspects of the branching pattern. This model comprises the following factors: the branching probability, the length of the dendritic segments, the branching angles and the spatial configuration of the branchings.

In order to establish an explanatory hypothesis about the branching of dendrites observed, it was next tried to find physical analogues. To this end the dendrites were considered as a system of pipes in which the cytoplasmatic flow is optimal with respect to energy cost. It appeared that a good correspondence existed between the configuration in such a system and the branching pattern of dendrites. As it is well-known that there is a flow of cytoplasm in growing dendrites, it is possible that this flow is an important factor in the final shape of the dendritic pattern. In the future more physical analogues will be considered.

An analysis of the length of the dendritic segments led to the hypothesis that the higher order segments of dendrites of neurones in the C.N.S. in adult mammals still have the capacity to form new branches. This has interesting possibilities with respect to memory, learning and the adaption of the C.N.S. to functional demands. To obtain more direct data on this point, an investigation was started in which the dendritic pattern of cortical neurones of adult rats that have been reared for a certain time in an enriched environment is compared with that of rats living for some time in isolation.

P. Kenemans:

An investigation concerning the macro- and microstructure of the brain stem of the 'ancient' bony fish *Polypterus* and of the closely related *Calamoichthys* was originally planned as part of a much wider comparative neuroanatomical workgroup study.

It was the aim of this group to start neuroanatomical comparisons between well-chosen vertebrate species, but only after developing a method of representation of neuroanatomical data which allowed meaningfull and reproducible results.

Therefore, after completing the descriptive and quantitative analysis of the nuclei of the cranial nerves, the cells of the reticular formation, of the cerebellum, and also of the ascending and descending fibre systems, much attention was given to methodological problems.

A topological method, based on ontological and functional anatomical principles, was tested on its accurateness and its limitations. Some intra-species comparisons were made with the aid of computer techniques. Next year a start will be made with interspecies comparisons.

E. van der Wiele:

Investigations on the peripheral olfactory system of the turtle (*Testudo hermanni*) have been finished (see Progress Report 1971). A publication is in preparation. This will contain a qualitative light-microscopical description of the olfactory and vomeronasal epithelia as well as a quantitative section in which, apart from the direct measurements, emphasis is laid upon the methodological problems which arise in a quantitative analysis performed with the light microscope.

K. C. Hodde:

Work on the analysis of the brain stem in lower vertebrates has been continued, with special reference to cartilaginous fishes. A survey of the existing literature is in preparation. This will accentuate the comparative point of view prevailing.

Data derived from a topological analysis of the brain stem have been obtained, which will be published in combination with similar analyses of brain stems from other lower vertebrates.

Work on the inventory of microscopic elements present in the spinal cord of dogfish was continued. A paper on the results obtained in an experimental investigation of ascending fibre systems by means of total transection of the cord and the degeneration technique, is in preparation. The main fibre system dealt with is the tractus spinocerebellaris with its various types of cerebellar endings.

In order to have a more accurate method available for detailed investigation in parts of the cerebellum as well as in the spinal cord, a combination of Nauta-staining and cryostat-technique has been developed.

3. SECTION OF ELECTRON MICROSCOPY

Dr. H. J. Romijn:

The results obtained in a light- and electron microscopic investigation on the rabbit pineal gland under normal and experimental conditions, performed during the past two years (see Progress Reports 1970 and 1971), were published in a thesis. The organ receives an orthosympathetic as well as a parasympathetic innervation. Bilateral extirpation and bilateral decentralization of the superior cervical ganglia revealed that most of the pineal postganglionic cholinergic nerve fibres, if not all, originate from intrapineal neurones while the preganglionic parasympathetic fibres course via the superior cervical ganglia to the gland. The cells of origin of the latter fibres are still unknown. The pineal postganglionic noradrenergic nerve fibres arise from nerve cells located in the superior cervical ganglia.

The hypothesis was put forward that in the rabbit pineal gland the synthesis of the pineal-specific compound melatonin occurs in two compartments, *viz.*, the preliminary serotonin synthesis in the light pinealocytes and the final conversion of serotonin to melatonin in the dark pinealocytes. Serotonin is probably synthetized by the smooth endoplasmatic reticulum in the terminals of the processes of the light pinealocytes and subsequently stored in small grey vesicles pinched off from this reticulum.

Recent experiments have given some indication with regard to the function of the pineal parasympathetic innervation. Submorphological alterations evoked in the pineal parenchyma after bilateral superior cervical ganglionectomy were compared with the results obtained after chemical orthosympathectomy by means of 6-hydroxydopamine. The results obtained led to the supposition that the release of the vesicle-stored serotonin from the terminals of the processes of the light pinealocytes into the intercellular and perivascular spaces is probably induced by the pineal parasympathetic cholinergic nerve fibres.

Finally, the mechanisms supposed to underly the neural control of the endogenously regulated serotonin rhythm and the exogenously controlled melatonin rhythm in the pineal of nocturnal mammals, could be integrated into a general working hypothesis.

Assistance was given to the investigations performed by Dr. M. A. Corner and H. A. A. de Jong (Section of Comparative and Developmental Physiology), Dr. B. Oestreicher and C. van Leeuwen (Division of Neurochemistry) and G. Boer (Section of Histochemistry and Cytochemistry).

Dr. J. M. Varela:

During the past year, work has been done concerning some problems of molecular neurobiology and ultrastructure of the brain stem reticular formation (BSRF). As for molecular neurobiology, an attempt has been made to disclose the functions of acetylcholinesterase (AChE) isoenzymes.

It is now generally accepted that AChE is the major mechanism of activation of the chemical mediator in cholinergic synapses. However, the existence of at least three AChE isoenzymes raises the question as to whether such multimolecular enzymic forms display differential patterns of degradation of the transmitter and therefore may be linked to different metabolic pathways with distinct physiological significance or may underline an unsuspected mechanism of fine regulation of synaptic events.

The following preliminary hypothesis was put forward: Each AChE isoenzyme is somehow related to one of the main ions implicated in the cholinergic synaptic transmission (Na^+, K^+, Cl^-) or, in other words, there is a specific differential involvement of AChE isoenzymes in the Na^+, K^+ and Cl^- conductances.

The matter was approached in an indirect fashion. One of the strategies has been to investigate the patterns of AChE zymograms of human, rat and dog red blood cells (poliacrylamide gel).

It is, indeed, well known that human red blood cells have a high K^+ and low Na^+ content, while the reverse is true for the dog red blood cells. Permeability changes in red blood cell membranes have been shown to be similar to those occurring in neurones. On the other hand, in both nerve and red blood elements the presence of distinct single pumps for Na^+, K^+ and Cl^- has been demonstrated.

Therefore, if the above hypothesis is correct, *i.e.*, if there is one AChE isoenzyme for each ionic pump (or ionophore), one should expect the electrophoretic patterns for AChE of the human and dog blood cells to be dissimilar and to reveal one isoenzyme predominating in one species and another one abounding in the other. This, in fact, turned out to be the case. Of course one is aware, that the evidence that in red blood cells with high Na^+ values the predominant AChE isoenzyme differs from the one which abounds in the high K^+ red blood cells, though suggestive, does not prove any definite relationship between the isoenzymes and the

ions. The issue is now tested in rat brain by using the substances which have shown selective effects on the fluxes of these ions (tetraethylammonium for K^+, hexametonium for Na^+, etc.)

The fractioning of AChE into isoenzymes by gel chromatography using Sephadex G-200 having succeeded, the search for actual distinct reactions of these isoenzymic forms towards such drugs has now been started. Other experimental procedures (histochemistry, electrophoresis) to test the above issue have also been used.

The study of actual differences in the isozymic patterns of AChE in muscle and brain has been regarded as another path to lead to information about differential roles of these isoenzymes. Such differences were found in poliacrylamide gel. A large amount of an isoenzyme appears to be present in the muscle which is different from the one predominating in the brain.

It has also been possible to demonstrate in this investigation that brain and muscle acetylcholinesterases of rat differ as to temperature and pH optima, Km and saturation curves, for example. The electrophoretic observations thus appear to match the kinetic data.

The next step will be to characterize the individual isoenzymes of both tissues on the same kinetic grounds. After this has been done, a second assumption will be considered in which a different subcellular localization for each isoenzyme was postulated (Varela, 1969). The findings already in hand allow significant insight into the way AChE works in the peripheral (muscle) and central nervous system junctions, and permit some tentative proposals about the differential roles of its isoenzymes. On this subject two papers are in preparation.

As to BSRF (brain stem reticular formation), in rabbit some aspects were redefined which have already been investigated in the rat. Studies using light and electron microscopy have confirmed and extended previous reports. The morphological structure of the reticular core observed may be characterized as follows: 1) isodendritic neurones; 2) dendrites with few or no spines at all; 3) absence of short axoned (Golgi type II) nerve cells; 4) presence of a great number of axosomatic synapses chiefly surrounding the large elements; 5) lack or paucity of serial axo-axonal synapses; 6) presence of a conspicuous amount of thin calibre and finely myelinated fibres; 7) existence of a dense interstitial plexus formed by afferents from various sources and by short and long efferents the reticular units send to each other. Some of these connections are established with reticular neurones of the opposite side.

Differences between the medial and the lateral portions as well as between the various levels of the BSRF were found. For example, axosomatic synapses are more frequent around the big or giant cells of the medial RF, whereas they are less common or rare and outnumbered by axodendritic contacts in the external part of the core where the small neurones predominate. Cells in the two reticular segments could also be

distinguished by the features of their endoplasmic reticulum. Electron micrographs revealed also a scarceness of axosomatic synapses from the midpons upwards, a finding which was related to the decrease of the big cells in the BSRF at more rostral levels and which appears to indicate differences between individual levels of the core disclosed by other techniques.

Finally, ultrastructural research of both rat and rabbit BSRF has unravelled synapses on cellular structures which as yet could not be defined either as glial or dark ganglionic elements such as described by Szentágothai and others in some neuronal populations. The physiological relevance of these ultrastructural findings is discussed in a paper in press.

4. SECTION OF NEUROHISTOCHEMISTRY AND NEUROCYTOCHEMISTRY

Dr. J. F. Jongkind:

The investigations on substrate utilization of the embryonic chick brain, reported in the Progress Reports 1970 and 1971, were extended by measurements under different environmental conditions. Incubation of the embryos in an atmosphere of 100% oxygen did not produce any changes in the utilization of glycolytic substrates. This can be explained partly by the presumed anaerobic character of embryonic brain metabolism.

Experiments to determine the redox state of the embryonic chick brain during development were done with the help of the cytoplasmic and the mitochondrial redox couples: pyruvate/lactate and acetoacetate/beta-hydroxybutyrate, respectively. Although the pyruvate/lactate ratio indicated a high free cytoplasmic $NAD/NADH_2$ ratio, the acetoacetate/beta-hydroxybutyrate ratio pointed to a highly reduced state of the mitochondrial free pyridine nucleotides.

Whether the results obtained with the techniques on embryonic hemispheres are due to a metabolic inhomogeneity of the hemispheres will be investigated using ultramicrochemical techniques on parts of the hemispheres such as the ectostriatum, the ependymal proliferation zone and the choroid plexus.

The quantitative determination of neurotransmitters with the new fluorimetric method using cryostate sections (see Progress Report 1971) was further developed and the conditions under which the fluorescent compound was formed were investigated using rat hypothalamus and hypophyseal intermediate lobe as model systems. It appeared, that the reproducibility of the reaction gained by treating the frozen-dried sections in a gas-free environment with completely dry paraformaldehyde. The velocity of the condensation reaction could be directed by different condensation times and temperatures.

Dr. D. F. Swaab:

Within the framework of the FUNGO group "Regulation of hypophyseal functions", the physiology and pathology of neuroendocrine systems in-

volved in diuresis, growth and reproduction were studied in rat. It was, moreover, tried to solve some methodological problems met during this investigation. A comprehensive study of the literature on this subject and its methodological problems was published.

In order to allow an approximate microchemical determination of *in vivo* levels of biologically labile compounds, such as ATP, lactate, glucose, pyruvate and glycogen, in anatomically uninjured cortex and hypothalamus, two surgical methods were developed. No fundamental difference was observed either between the cortical and hypothalamic levels of these substrates, or in their fluxes. In collaboration with K. Boer (Section of Comparative and Developmental Physiology), the substrate fluxes during ischaemia were correlated with electrical activity in rat cortex and hypothalamus, recorded by means of telemetrically transmitted electroencephalograms. Following decapitation, electrical activity declined precipitously after 9.6 sec in the cortex, and after 12.1 sec in the hypothalamus. High levels of glycogen, glucose and ATP were present at this moment, while P-creatine had declined sharply.

In order to allow recording of the electrical activity of the hypothalamo-neurohypophyseal system (HNS) during reproductive stages like coitus and parturition in the freely-moving animal, K. Boer is developing a telemetric technique in collaboration with our Section. Results obtained by means of this technique, some of which have been discussed before, will soon be published and are also dealt with in the progress report of the Section of Comparative and Developmental Physiology.

In the last few years gonadotropic hormones were shown in our Section to have a direct stimulating effect on the synthesis of posterior lobe hormones. In collaboration with G. J. Boer we are now investigating the effect of gonadotropic hormones on the posterior lobe by means of enzymatic parameters and the effect of gonadotropic hormones on the water intake and urine production as measured by means of metabolic cages. Some preliminary results are in press.

In connection with the possible role of the HNS and gonadotropic hormones in the origin of toxaemia during pregnancy, the effect of vasopressin and gonadotropic hormones was studied on fluid intake, urine production, albuminuria, blood pressure and length of the gestation period in rat. This study was performed in collaboration with H. Veerkamp, a student in biochemistry. During the last pregnancy week fluid retention was observed in control rats. Vasopressin did not influence the gestation length, while it appeared to be possible to increase pregnancy length with at least 6 days by means of HCG. Vasopressin induced, in the doses used, a slight increase in bloodpressure and increased proteinuria; however, no fluid retention was observed. HCG did not influence these magnitudes. A more extensive study of this subject seems necessary.

In collaboration with W. J. Honnebier (gynaecologist at the Clinic for Obstetrics and Gynaecology, University Hospital of Amsterdam), a project

was started about the role of the foetal brain on intra-uterine growth of the foetus and in parturition. This theme is partly studied on human foetuses having no hypothalamus (anencephalics), and partly experimentally in rat. A simple technique has been developed to remove the entire foetal brain *in utero*. The foetal brain, in rat as well as in the human, appears to have an important influence on the growth of the foetus and its placenta. The role of the foetal neuroendocrine factors in labour was studied by means of intra-uterine injections in anencephalics and in rat bearing brainless foetuses. The results of these investigations have already partly been published.

During a graduate training in the general practice of L. G. Fransman, questionnaires were used in an attempt to establish the reactions of patients to the presence of a trainee. Only one percent of the patients proved to object to the presence of the trainee, while two percent marked their questionnaire for objection as well as for no objection. This study has been published and will be continued by the "Instituut voor Huisartsgeneeskunde" (Head: Prof. B. S. Polak).

G. J. Boer:

The investigation into the role of the pituicytes (glial cells) in the neuroendocrine hypothalamo-neurohypophyseal system (HNS) was continued (see Progress Report 1971).

According to Kurosumi (1971), the pituicyte would function as a phagocyte in order to remove materials, left behind after release of hormones from the axonal endings in the neural lobe. Using quantitative microchemical techniques (Lowry, 1953), the activity was measured of those enzymes which might be the tools for the function of the pituicyte as phagocyte, namely the lysosomal enzymes.

As a continuation of the investigations in 1971 studying the results after water deprivation (Boer and Jongkind, publication in preparation), this year the activities of the lysosomal enzymes acid phosphatase, β-glucuronidase, β-galactosidase, β-glucosidase and arylamidase N in the rat neural lobe were determined under other experimental and physiological conditions resulting in known or expected enhancement of the release of neurohypophyseal hormones, *i.e.* during parturition and lactation and following gonadectomy (in collaboration with Dr. D. F. Swaab). In all conditions mentioned, an increased lysosomal activity of the neural lobe tissue was found just like during osmotic stress, but of a somewhat different pattern.

Since we do not know whether these different responses are either of axonal or of pituicytic origin, histochemical techniques were again tried out in collaboration with Dr. J. F. Jongkind in order to localize these changes. Only a new technique of Meyer (1972) for acid phosphatase gave any result as to this localization, but, here, no change in histochemically demonstrated enzyme activity could be found.

Because both enzyme histochemistry and electronmicroscopy seemed to fail in the localization of measurable changes in lysosomal activity, experiments were started for a microbiochemical separation of axonal endings and pituicytes.

Division of Physiology

1. SECTION OF EXPERIMENTAL NEUROLOGY

C. de Blécourt, B. van Cranenburgh, W. van Emde Boas, J. P. Muizelaar, and Dr. J. P. Schadé:

Research concerning the spontaneous neuronal activity, the iontophoretic application of neurotransmitters and EEG-analysis in normal and experimental atherosclerotic rabbits was continued.

Spontaneous neuronal activity

The classification to depth of the recorded neurones was adapted in such a way that all neurones were classified in one of 6 depth classes, each comprising 3000 μ. In this way it was possible to analyse separately *i.a.* cerebral cortex, white matter and the basal ganglia.

The activity recorded was analysed and comprised in the form of interval histograms. From these the following parameters have been computed: mean firing interval, standard deviation of mean firing interval, coefficient of variation, gamma-parameter, mode and skewness. The interval histograms were also classified according to a number of objective criteria as to shape into four types: exponential-, gamma-, gaussian- and multimodel type.

The distribution of these four types was found to differ significantly between the normal and the atherosclerotic experimental series. In the normal series this distribution differed also significantly between the different depth classes; in the atherosclerotic series this difference in the distributions, dependent on depth from the cortex, was not observed, indicating a loss of specificity of the neuronal firing patterns in the atherosclerotic animals.

The means and standard deviations of all parameters were computed both for all series as a whole and for all depth classes separately. Statistical significance was established according to the Kruskal and Wallis one-way-analysis of variance test.

Significant differences were predominantly observed in the basal ganglia and the cortex. They include a lower mean interval, smaller standard deviation and coefficient of variation, and a higher gamma parameter in the atherosclerotic series, indicating a decrease in variability of the neuronal firing patterns under influence of experimental atherosclerosis.

The correlation between each of the six parameters relative to all others was computed. A high correlation was found to exist between the mean interval and the standard deviation, both in normal and in experimental

atherosclerotic animals, although there is a slight difference between the series.

Quantitative analysis using a correlation matrix is under way. Frequency histograms for all parameters have been made as to depth class as well as for the series as a whole to establish any differences in neuronal populations and their possible changes due to the effect of atherosclerosis.

Morphological studies on preparations of both normal and atherosclerotic animals are now made to establish the pathological anatomy of the blood vessels and brain in atherosclerosis, and the exact electrode track and the recording sites in a series of rabbit brains.

A detailed and extensive survey of the literature is being performed concerning statistical analysis of neuronal activity in different brain areas and different animal species; atherosclerosis in general, and the relation between experimental atherosclerosis in animals and clinical atherosclerosis in man; pathological, neurological and psychiatric aspects of cerebral atherosclerosis in man and its relation to general ageing processes in the human brain.

In general, the literature seems to support our choice of experimental model, our results and our working hypothesis on the selective vulnerability of certain, mostly inhibitory neuronal systems in the mammalian brain to degenerative processes such as general atherosclerosis.

Iontophoretic application of neurotransmitters

By means of iontophoresis, acetylcholine, serotonin, dopamine, and noradrenaline were tested in normal and atherosclerotic rabbits. Iontophoretic application of acetylcholine and noradrenaline showed significant results.

EEG changes

Significant changes were found between normal and atherosclerotic animals in the low-frequency (1–4 Hz) band of the EEG. A close relationship exists between the plasma-cholesterol level and the increase in low frequency EEG-activity. An inverse relationship is present between the latency time and negative spikes of the visually evoked potential.

All these data point to a differential vulnerability of the various synaptic inputs of central neurones. Our results indicate that, in the near future, it will be possible to unravel the physiological mechanisms underlying degenerative patterns caused by experimental atherosclerosis.

Dr. J. C. de Valois, Dr. J. P. C. Peperkamp, C. V. de Blécourt, and P. A. de Groot:

The investigation of experimentally induced cerebrovascular disorders in the rabbit was continued. The cerebral blood flow (CBF) has been measured using the almost classical intra-arterial injection technique with the inert gases 85-Krypton and 133-Xenon.

A thesis, based upon investigations of the cerebral blood flow in the rabbit under normal physiological conditions, during anaesthesia and after administration of vasoactive drugs, was published (Dr. J. P. C. Peperkamp). The results can be summarized as follows.

The intra-arterial injection technique (LASSEN and INGVAR, 1963) appeared to be most suitable for quantitative determinations of the cerebral blood flow. After injection of the isotope – 133-Xenon or 85-Krypton – into the internal carotid artery, the clearance of the isotope from the brain tissue can be registered by means of one or more scintillation detectors placed over the intact skull. From the clearance data obtained the cerebral blood flow can be calculated.

In a series of experiments, the cerebral blood flow was determined under normal conditions in non-anaesthesized animals. The variability of the cerebral blood flow in two consecutive determinations in one single animal was 4.7%, while the variability in the cerebral blood flow between various animals was 19.3%. The average cerebral blood flow in the rabbit under the experimental conditions used appeared to have an average value of 44.8 ml per 100 g brain tissue per minute (SD. 8.7 ml/100/g/min).

As CO_2 influences cerebral blood flow, the effect of CO_2 on the cerebral blood flow was determined. A change in the arterial CO_2 tension of 1 mmHg resulted in an average change in cerebral blood flow of 0.6 ml per 100 g brain tissue per minute.

The influence of the following anaesthetics on the cerebral blood flow was determined: Nembutal, Kemithal, Hypnorm, halothane and halothane with nitrous oxide. In general, these anaesthetics reduce the cerebral blood flow for about 20%.

Measurement of the cerebral blood flow during 5–6 hours revealed that even short-acting anaesthetics lower the cerebral blood flow over a long period of time. When using these anaesthetics, the control cerebral blood flow values are not reached within this measuring time (5–6 hours).

In the literature, conflicting results concerning the effects of halothane on the cerebral blood flow are reported. Unsupplemented halothane (½%) anaesthesia appears to lower the cerebral blood flow. Combining halothane with nitrous oxide results in a significant increase in cerebral blood flow of about 15%.

The effects of the following vasoactive drugs on the cerebral blood flow were also investigated: Hydergine, Duvadilan, Duspatal, papaverine, Ronicol, Cyclospasmol and nicotinic-acid. Comparison of the effects of these drugs on the cerebral blood flow with the control series did not show any significant increase in cerebral blood flow, despite injection of relatively high doses into the internal carotic artery. Nicotinic-acid (pH 3.6), Ronicol and Cyclospasmol even significantly decreased cerebral blood flow.

The reactivity of the cerebral blood vessels was tested in an additional series of experiments in which Metrazol and nicotinic-acid (pH 7.2) was

administrated. After injection of Metrazol the mean cerebral blood flow increased to 98.4 ml per 100 gram brain tissue per minute.

A project was undertaken to find a relationship between local CBF and local EEG using photic stimulation to change the electric activity in the occipital lobes (C. V. de Blécourt).

In order to investigate the circulatory effects of a number of experimentally induced cerebrovascular disorders (f.i. arachnoidal haemorrhage, vasospasms, embolia and atherosclerosis – the latter in collaboration with Dr. J. P. Schadé –), a method has been developed to measure regional cerebral blood flow values (rCBF values) in the rabbit (P. A. de Groot). This method is derived from the autoradiographic techniques originally developed by Kety and Landau and modified by Reivich and Eklöf. 14-C antipyrine was injected intravenously. According to the Fick principle the amount of this tracer in the cerebral structures is a measure of the blood flow through them. The measurement of the radioactivity in the brain will be performed by means of autoradiography and direct counting in a liquid scintillation counter. The results of these measurements yield quantitative values for rCBF in ml/g/min.

A comparison of this method will be made with the intra-arterial injection technique using 85-Krypton or 133-Xenon which has been used in our Institute for the past four years. A number of technical problems concerning the operative techniques to induce experimental (sub-)arachnoidal haemorrhage and spasms of the basilar arteries have already been solved. These experimental studies are meant to form the basis for future clinical investigations in coöperation with the Neurosurgical Clinic of the University of Amsterdam (Head: Prof. Dr. W. Noordenbos).

A thorough investigation was carried out on the macroscopic and microscopic aspects of the cerebral vascular beds using the Spalteholz technique and microradiography (Dr. J. C. de Valois). This study has been performed in the Clinic for Experimental Surgery of the University of Amsterdam (Head: Dr. J. P. Klopper).

In co-operation with the Neurosurgical Clinic of the University of Amsterdam (Head: Prof. Dr. W. Noordenbos), CBF measurements are started in cases of subarachnoid haemorrhage (Dr. J. C. de Valois). To avoid carotic puncture, techniques will be worked out to investigate the possibility to use intravenous injection of the isotope and, subsequently, three compartmental analyses of the clearance curves.

2. SECTION OF COMPARATIVE AND DEVELOPMENTAL PHYSIOLOGY

Dr. M. A. Corner:

In collaboration with the Section of Histochemistry and Cytochemistry the question has been pursued of the possible role of glutamic acid in the normal development of cerebral bioelectric activity in the chick embryo. The critical period of electrocortical (EEG) development, namely,

coincides with a sharp rise both in glutamate-metabolizing enzymes and in the concentration of glutamate in the brain tissue. Moreover, previous findings from this Institute, using the convulsant drug methionine sulphoximine, strongly indicated that glutamatic acid metabolism changes dramatically at precisely the stage of development at which the cerebral EEG becomes mature. Establishment of a causal relationship would mean either that glutamate is an important neurotransmitter in the avian cerebrum, or else plays a less specific role in modulating the excitability of the network as a whole.

In accordance with expectations based upon the above hypothesis, injection into younger embryos of sufficient glutamate to raise the cerebral concentration to that measured just after EEG maturation, caused the cerebral EEG to take on an appearance typical of later stages. As in normal ontogeny, the change consisted of large amplitude slow wave complexes appearing at variable intervals, superimposed upon the higher frequency background electrical activity. In some cases the post-glutamate EEG consisted of an almost continuous train of these large potentials, thus mimicking the essentially mature bioelectric pattern. Since such an experimental simulation of the normal developmental changes is a necessary but not sufficient condition for the postulated causal role of glutamate to be established, a specific antagonist against the functional effects of this substance was sought. The literature suggested only two good possibilities, one of which – administration of glutamic acid diethylester – was then tested upon the above-mentioned preparation. Injection of this substance indeed caused the large amplitude slow waves to disappear from the cerebral EEG of late embryos, leaving only smaller background potentials as present in the early functional stages.

The control and experimental EEG records were subjected to a quantitative treatment (broad-band frequency analysis) in order to look for more precise correlations between the degree of cerebral functional maturation and the measured level of glutamate or other substances in given individuals. The quantitative analysis is now being pursued further, using higher-resolution techniques, in collaboration with the Section of System Analysis.

The project of developing a self-sustaining nerve tissue culture set-up for electrophysiological studies has been continued, and some improvement in the survival of whole embryos (chick) cultured *in vitro* has been achieved. Such a technique is of interest because of the more closely normal physiological conditions under which the isolated neurones or groups of neurones develop. Studies of such tissues would be an important control upon the relevance of *in vitro* studies using classical methods for the understanding of developmental processes in the whole organism. Although routine culture is already possible for 1–2 weeks using this method, it is still preferred to improve the host culture system before starting to apply it to the investigation of isolated tissues. Furthermore, specific neural

staining techniques, vascular perfusion and electron microscopy have been worked out for suitable application to the developing chick brain, within the framework of the above project.

A portion of the year was spent working at the Stazione Zoologica in Naples, under auspices of the Royal Netherlands Academy of Sciences. The aim of this project was to compare the early stages of behavioural development in a neurologically highly evolved invertebrate with what is known about vertebrate embryonic movement patterns. This is important in the search for possible universal features in the functioning of primitive neuronal networks. In both the squid and the cuttlefish (Mollusca, Decapoda) spontaneous motility is highly developed. As in vertebrates, it begins with isolated and irregular twitches in various parts of the body, and later becomes periodic in nature. There are also some indications for the occurrence of stereotyped motor discharges of maximal intensity, such as characterize the early behaviour of all vertebrate embryos which have been studied to date.

As in certain vertebrate classes (Mammalia, Amphibia) the first reflex movement can be elicited already at the time of the earliest spontaneous movements, in contrast to birds and fish, where sensory development lags behind motor development. What seems basically different in cephalopod embryos as compared to vertebrates is the persistent lack of integration among various muscle groups even during the most vigorous spontaneous or reflex movements. 'Total body' motor co-ordination in these animals is confined to the mantle – together with fins, funnel and collar – with the chromatophores and head-arm-tentacle musculature contracting sporadically and largely independently. Behaviourally tested material was fixed for later neurohistological and electron microscopic study, in relationship to the question of the structural basis of the above observations.

W. L. Bakhuis:

Part of the (1971) doctoral work was chosen to be worked out for a Ph. D. thesis, namely the nervous control underlying stereotyped motor behaviour. However, the material used until now, *i.e.*, the chick, was not suitable because this would require time consuming film analysis, and also because one would have very serious difficulties in immobilizing the head sufficiently to allow intracellular work for the investigation of behaviour (hatching).

Last year, during a preliminary research with Cephalopodes (Mollusca), is was found that both of the above-mentioned problems would be minimal if these animals were chosen as experimental material. Cephalopodes show a type of motor behaviour, *i.e.* colour display, which is relatively easy to analyse and does not involve disturbing body movements. By means of the thousands of chromatophore organs in their skin, these animals can produce many different colour patterns expressing their motivational

condition or having a function in mimicry. Many of the patterns are static and can, therefore, readily be characterized. The different states of expansion of each pigment cell of a chromatophore organ are effectuated by the degree of contraction of the many muscle fibres surrounding the pigment cell. Since even rapid contractions of these muscles cause no visible movement of the overlying skin, it will be clear that they can produce no body movement artifacts interfering with intracellular work in the central nervous system.

As it is not possible to work with Cephalopodes at this Institute, it was decided to plan to do the necessary experiments abroad. During the summer it was possible to work at the Stazione Zoologica in Naples with the decapod molluscs *Sepia officinalis* and *Loligo vulgaris*. At the Caribbean Marine Biological Institute CARMABI on Curaçao an investigation was carried out showing that the decapod, *Sepioteuthis sepioidea* could be caught and maintained (adequate food appeared to be easily obtainable) during a long enough time to allow work with this animal.

The research planned will consist of the following three parts:

a) an investigation of the (chromatophore) areas innervated by the branches of the pallial nerve (*i.e.*, the connective between the central nervous system and the effectors of the mantle);
b) an investigation of the different ways in which chromatophores belonging to one motor unit can be arranged topographically;
c) an investigation of the functional hierarchical relations among the central neurones which bring a colour pattern about.

Enabled by a grant from the Royal Netherlands Academy of Sciences, a large amount of the necessary experimental work of part a) has already been done using *Sepia* and *Loligo* as material at the Stazione Zoologica in Naples. Different branches of the pallial nerve were severed after which the effects of the cuts were examined. Some contradictions in the literature could be explained. A number of the experimental animals were kept alive for 2 days following the operation. Then they were sacrificed and fixed for degeneration studies of the fibres running between the stellate ganglion and the fin nerve.

Research will be continued at CARMABI during the winter using *Sepioteuthis sepioidea*. The project will emphasize finely localized electrical stimulation by means of micro-electrodes.

K. Boer:

The major aim of the present investigation, which is being carried out in collaboration with Dr. D. F. Swaab of the Section of Histochemistry and Cytochemistry, is to correlate neuronal firing in the supraoptic nucleus (SON) of the unanaesthetized, freely moving female rat with motor reactions during delivery of the young.

Multiple-unit neuronal activity in the SON will be chronically recorded using a bipolar semi-microelectrode, by means of which a sufficiently high signal to noise ratio can be obtained that persists even after several days to weeks. Development of such an electrode system is now nearly completed.

For the implantation, a motorized stereotactic set-up was worked out in order to allow continuous monitoring of the neuronal firing during implantation, and thus the selection of an optimal recording site before cementing the electrode.

The signal recorded in this way is telemetrically transmitted by a specially built subminiature sender, placed directly upon the head of the animal. In this way, electrical movement artifacts as well as movement restrictions can be avoided. Since it is now established that an increase in electrical activity of neurosecretory cells of the SON indicates the release of posterior lobe pituitary hormones, the signal recorded can serve as an index for the release of these hormones. This means that correlation of the neuronal activity of the SON with motor patterns occurring during labour would provide information about the effects of the posterior lobe hormones upon this aspect of reproductive behaviour. In order to correlate such signals with the motor patterns one needs to be able to identify clearly the latter. An attempt to do so will soon be made by means of behavioural studies and registration techniques, such as electromyography and/or intrauterine pressure recording.

A difficult problem on the neurophysiological side is that of the precise localization of the recording tip in order to be sure that one is recording from neurones which project to the neurohypophysis. Antidromic stimulation of the SON by the way of the posterior lobe would eliminate the problem, and that technique is now being worked out.

An additional but related project, which is a continuation of earlier electroencephalographic studies, is the study of the sleep-waking patterns of bioelectrical activity within the hypothalamus. There seem to be no qualitative differences with the well-known basic cortical pattern, namely relatively high amplitude, low frequency potentials during sleep, with smaller potentials of higher frequency predominating during waking. A more specifically hypothalamic phenomenon in the rat, *viz.*, that of large regular thèta-waves during behaviourally highly alert states, has already been mentioned in an earlier publication with Dr. D. F. Swaab.

All these functional states, as revealed by macro-electrode registrations, are planned to be worked out further in conjunction with a micro-electrode study of the various neuronal discharge patterns in the same region of the brain.

Dr. H. van Wilgenburg and H. A. A. de Jong:

In order to investigate the membrane properties of neurones with determined input-output relations, studies were made of a group of neuro-

secretory cells in the left parietal ganglion of the snail, *Helix pomatia*. These cells, as could be demonstrated by guestworker R. Westerwoudt, receive dopaminergic input from identified neurones in the cerebral ganglia, while they probably release their neurosecretory substances to the heart. Using the voltage clamp technique to study the ionic mechanism, it was found that the increase of potassium permeability, by dopamine release, is enhanced by corticosteroids. The results obtained are now ready for publication.

The study on statocyst-evoked neuronal activity in the CNS of the snails *Helix* and *Aplysia* is also part of the research programme for analysing a relatively simple neuronal network.

Intracellular recordings from neurones in the cerebral ganglia of *Helix* were made by guestworker B. Huisman.

After a thorough preparation, H. A. A. de Jong, Dr. P. H. Oosting and Dr. H. van Wilgenburg spent a six week's stay at the Stazione Zoologica, Naples, supported by the Naples Commission of the Royal Netherlands Academy of Sciences, to study the statocyst-evoked neural activity of *Aplysia*. The data stored on magnetic tape will learn more about the primary sensory cells, the integration capacity of the interneurones, and the relation of their output to behaviour.

A computer programme has been developed by the guestworker L. Sauren to stimulate the equilibrium system of the snail.

3. SECTION OF BEHAVIOURAL PHYSIOLOGY

H. van Dis, N. E. van de Poll, B. Bermond, and M. H. Roest:

Investigations on the differentiation of the neural substrate for male and female sexual behaviour were further elaborated. Use has been made of various techniques: homologous and heterologous hormone manipulations, intracerebral implantation of hormones, lesions, electrical stimulation and pharmacological treatment.

An intra-individual comparison was made between the changes in male sexual behaviour following castration on the one hand and during sexual exhaustion on the other. Further experiments have been carried out on the role of androgens on sexual exhaustion in male rats. The results will be published.

The role of testosterone, oestrogen and progesterone on male and female sexual behaviour has also been studied. It was shown that testerone is important for the exhibition of female mating patterns in male animals. The results were prepared for publication. The effect of implantation of crystalline testosterone-propionate into the preoptic area and the anterior hypothalamus upon male and female mating behaviour was studied in neonatally and adult-castrated male rats.

A series of experiments was started on the effects of medial preoptic

and anterior hypothalamic lesions on both male and female sexual behaviour.

In the past, the use of actively mounting "stimulus males" in the testing of male rats for female mating patterns was often a methodological problem. In a replication study it was shown that male rats treated with testosterone and para-chlorophenylalanine exhibit excessive and compulsive mounting behaviour, and are therefore very useful as "stimulus males". Furthermore, a study was performed on the effect of para-chlorophenylalanine on the mounting behaviour in ovariectomized female rats. The results will be soon published.

The preliminary experimentation with the new equipment for intracranial self-stimulation was completed. A technical note is in preparation.

Further experimentation on the effect of olfactory bulb ablation on maternal behaviour was carried out. A manuscript is in preparation.

Studies on agressive behaviour have been partially supported by the Brain and Behaviour project "Aggression". Various ways of inducing aggressive behaviour in Wistar rats have been tried, and a comparison was made with the aggressive behaviour of a different strain of rats (S 3). It was shown that isolation-aggression could be induced in S 3-rats. A series of experiments was started on the role of androgens in the development and maintenance of aggressive behaviour patterns.

In collaboration with Prof. Knut Larsson, University of Göteborg, Sweden, a theoretical paper on sexual behaviour is in preparation.

Studies on autonomic responses in human male and female subjects during sexual arousal were further elaborated in collaboration with M. A. de Jong, Department of Psychophysiology, University of Amsterdam.

J. P. C. de Bruin:

Research on the influence of telencephalic lesions on the various behavioural patterns of the Siamese fighting fish (*Betta splendens*) has been continued (see Progress Report 1971).

Aggressive and sexual behaviour have been analysed within three main categories: approach-withdrawal, lateral display and frontal display, which are each composed of smaller behavioural units. It was shown that the lesions are greatly influencing aggressive and sexual behaviour. Especially the components of aggressive display will change dramatically, depending upon size and site of the lesion. Some components will increase in frequency and/or duration, while others will decrease or remain unchanged. This necessitates a quantitative description of the behavioural categories under study consisting of many parameters.

Sexual behaviour can be manipulated as well, although the changes caused by the lesions are less pronounced than in aggressive behaviour. The exact site of the lesions are studied in histological sections.

In a study in which the display-evoking stimulus is offered repeatedly,

habituation may occur. Habituation is defined as a waning of the response, due to repeated previous application of the stimulus. A separate study was undertaken to investigate when habituation of the aggressive response would occur and how the phenomenon may be characterized. It has been shown that no habituation of the aggressive response will occur in the present experimental set-up.

A. P. van der Meché and T. Röthengatter:

Memory formation was studied in the goldfish (*Carrassius auratus*) with the aid of various learning models: one-trial learning, active avoidance conditioning, and conditioning with positive reinforcement.

Further experiments have been performed on one-trial learning, using an intra-individual design. This experimental design is more appropriate for the pharmacological approach of this behaviour. The effect of electroconvulsive shock upon memory formation in the one-trial learning situation was investigated. Treatment of the goldfish with cycloheximide, a protein-synthesis inhibitor, 30 minutes before the learning trial did not influence memory formation in this situation.

From an analysis of the inter-trial responses during active avoidance conditioning experiments the following hypothesis was derived. Intertrial responses in the goldfish have to be considered as a remainder of spontaneous responses. The small amount of inter-trial responses as compared to the spontaneous rhythm is due to an inhibiting influence of punishment on barrier crossing, during conditioning experiments. This hypothesis has to be tested using conditioning with positive reinforcement.

A series of experiments has been started on the effect of ACTH and dexamethasone on the active avoidance conditioning in the goldfish.

The development of a new equipment for conditioning with positive reinforcement in the shuttlebox was completed.

4. SECTION OF NEUROPHYSIOLOGICAL SYSTEM ANALYSIS

Ir. J. Smith and W. J. J. Houtzager:

Results were obtained in connection with the projects mentioned in detail in the Progress Report 1971.

a. *The cellular level.*

The firing characteristics of single neurones are still being studied in collaboration with the Section of Experimental Neurology. The histogram, the mean, the standard deviation, the coefficient of variation, the gamma parameter, the mode and the skewness of the interspike intervals appeared to be representative parameters for spontaneous neuronal spike activity. Some of the results obtained have already been published. Significant differences between control and atherosclerotic rabbits are found for these parameters, for all depth classes as well as for the individual depth classes.

A further classification of the functional aspects in different parts of the rabbit brain suffering from experimental atherosclerosis is in progress.

b. *The neuronal population.*

Biochemical and electrophysiological investigations of the electrical recordings obtained from the chick embryo indicate a clear distinction between several "critical periods" in the cerebral electroencephalogram during prenatal stages. A study of these functional changes may elucidate the mechanisms of the generation and patterning of complex neuronal activity. In normal ontogeny, the main characteristics consist of large amplitude slow wave complexes appearing at variable intervals which are superimposed upon the higher frequency background activity. These developmental electrical changes cannot be satisfactorily analysed using the classical EEG-filtering techniques. They are therefore now subjected to the "aperiodic extreme amplitude-interval method". The results will be soon published. The above mentioned criteria will also be applied to the quantitative evaluation of the experimentally altered cerebral activity. This work is done in collaboration with Dr. M. A. Corner (Section of Comparative and Developmental Physiology) and with Dr. J. F. Jongkind (Section of Neurochemistry and Cytochemistry).

c. *Parts of the brain as a functional unit.*

The investigation concerning the analytical aspects of the cerebral blood flow (CBF) with Dr. J. P. C. Peperkamp is finished, resulting in a thesis of the latter. A three-compartmental description of the so-called inhalation method and of the intravenous approach to measure CBF is in progress. The use of 14-C-antipyrin necessitates a different mathematical treatment of the Fick-principle. This will be done in collaboration with the Section of Experimental Neurology.

An investigation concerning the functional connection between the EEG and the CBF is still in progress in collaboration with C. V. de Blécourt (Section of Experimental Neurology).

Division of Neurobiochemistry

Dr. A. B. Oestreicher and C. van Leeuwen:

Knowledge of the processes occurring during neurotransmission is derived, among other things, from studies on the interactions and properties of macromolecules, such as glycoproteins. These are structural components of nerve cells and can be isolated in synaptosomes, synaptic plasma membranes and synaptic vesicles. 5–15% of the total protein in rat brain consists of glycoproteins. This is bound for 70–80% to membranes, the

remainder being found in the soluble fractions. Cytochemical investigations have shown that glycoproteins occur rather abundantly in the synaptic complex.

In order to study the properties and interactions of glycoproteins occurring in the synaptosome, a fraction enriched in synaptosomes and fractions containing synaptic plasma membranes have been isolated from whole brain of the rat according to the procedure of Cotman and Matthews. The yield of protein from one gram wet weight of brain in, respectively, the synaptosomal fraction and the synaptic plasma membrane fraction, is 10 mg and 2 mg. The isolated fractions were characterized by: measurements of the activity of marker enzymes such as monoamine oxidase, acetylcholinesterase, lactate dehydrogenase, Mg-ATPase and (Na, K)-activated ATPase; measurements of chemical markers such as protein- and lipid-bound N-acetylneuraminic acid and lipid phosphorus; and by electron microscopy.

The isolated synaptic plasma membrane fraction contained (Na, K)-activated ATPase showing a specific activity of about six times as high as that present in the original homogenate. This finding agrees well with the results reported by Morgan, Wolfe, Mandel and Gombos.

The following methods for analysis of glycoproteins have been introduced at the Institute: polyacrylamide gel electrophoresis; thin layer and paper chromatography, in order to separate monosaccharides; and chemical determinations of N-acetylneuraminic acid, neutral sugars and fucose, and of hexosamines.

Future investigations will consist of: isolation of glycoproteins from the subcellular fractions obtained by means of differential extraction, followed by affinity chromatography and gel filtration; a study of these glycoproteins by means of the methods earlier mentioned; and a study of the function of the glycoproteins in the synaptic membrane with respect to the transport of metabolites into and out of the synaptosome.

Division of Neuropharmacology

Dr. J.-J. Meisch and M. van Wijk:

Sensitive methods for the separation and fluorometric determination of the false neurotransmitter 4-methyl-α-ethylmetatyramine (H 75/12) and its β-hydroxylated product have been set up. No *in vivo* β-hydroxylation of H 75/12 could be demonstrated in mouse and rat brain.

Pharmacodynamical studies with H 75/12 such as time and dosis response curves in various tissues and in blood plasma of the mouse and the rat have been nearly completed. The effect of various antidepressants on the false transmitter in question will be studied in the future.

Further technical progress with the separation and the fluorometric determination of serotonin, its precursors and its metabolites has been achieved. In the same brain sample the antidepressant drugs imipramine and desipramine, its desmethylated derivative, can be measured. The acute and the chronic effect of these antidepressants on serotonin metabolism in rat brain will be compared.

J. ARIËNS KAPPERS

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HUBRECHT LABORATORY

International Embryological Institute – Utrecht

PROGRESS REPORT 1972

History and objectives of the institute

The Hubrecht Laboratory was founded in 1916 in memory of the Utrecht zoologist and embryologist Prof. A. A. W. Hubrecht. It is a non-governmental institution operating under the supervision of the Royal Netherlands Academy of Arts and Sciences.

The objective of the Laboratory is to function as an *international research and service centre for developmental biology*. To ensure a multidisciplinary approach to the many problems of development eight research units applying a variety of experimental approaches have been established (see under Scientific Staff below).

The Laboratory aims at stimulating international co-operation and understanding by, among other things, organizing International Research Groups in Developmental Biology at more or less regular intervals, and by the biennial publication of an international directory of investigators active in developmental biology (General Embryological Information Service).

The Laboratory houses the Central Embryological Library (collection of reprints covering the field of developmental biology) and the Central Embryological Collection (microscope slides and material preserved in alcohol).

The Laboratory is the administrative seat of the International Society of Developmental Biologists.

Individual guest workers are welcome at the Laboratory. Partial financial support is available in special cases only.

Management and Scientific Staff

P. D. Nieuwkoop, Ph.D.—Director, Prof. of Experimental Embryology, University of Utrecht
J. Faber, Ph.D.—Deputy Director
B. Cool—Laboratory Manager

B. Z. Salomé, M.Sc.—Chief Librarian

J. G. Bluemink, Ph.D.—Research unit of ultrastructural research
Elze C. Boterenbrood, Ph.D.—Research unit of experimental morphology; Curator of the Central Embryological Collection
K. Hara, Ph.D.—Research unit of experimental morphology
Vacancy—Research unit of developmental physiology
S. W. de Laat, M.Sc.—Research unit of biophysics
Kirstie A. Lawson, Ph.D.—Research unit of tissue and organ culture
W. J. Ouweneel, Ph.D.—Research unit of developmental genetics
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Geertje A. Ubbels, Ph.D.—Research unit of histo- and cytochemistry

Seventh International Research Group in Developmental Biology — 1972

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The Research Group met from January 17th till July 15th, 1972. It had no central topic, and the members worked on a variety of subjects in close co-operation with individual staff members of the Hubrecht Laboratory. In most cases only preliminary results were obtained. The members whose names are marked with an asterisk stayed on at the Laboratory as visiting scientists after the official closing date of the Research Group.

Visiting Scientists – 1972

Madeleine Friant, Ph.D., M.D. (Paris, France)
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Alina Sutasurja, M.Sc. (Bandung, Indonesia)

Temporary Research Assistants – 1972

Th. F. J. de Greeff, M.Sc.
J. M. van der Meer, M.Sc.

Ph.D. students (University of Utrecht) – 1972

R. A. F. Dekker, M.Sc.
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Graduate students (University of Utrecht) – 1972

C. Inge M. Bänziger, B.Sc.
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(For Dutch workers B.Sc. and M.Sc. are used as the approximate equivalents of the Dutch degrees of Biol. Cand. and Biol. Drs.)

I. Early amphibian development

INTRODUCTION

Experiments carried out in this Laboratory during the past six years (see previous reports) have shown that important morphogenetic interactions occur in the amphibian blastula, i.e. before so-called "primary induction" starts. These findings have led to a renewed interest in these early stages, which is reflected in most of the work reported in this section.

A. EXPERIMENTAL MORPHOLOGY AND CINEMATOGRAPHY

1. *Further studies of the cleavage pattern (Ambystoma mexicanum)* (K. Hara)

1a. Cinematography

The analysis was continued of 15 pairs of films taken in preceding years by means of "double-camera" time-lapse cinematography (see report of

1969, sect. I.a.5). The duration of the cleavage cycles in the animal pole area is practically constant from the onset of the 3rd cleavage till the onset of the 10th cleavage in all embryos examined. This duration was taken as a unit in which to express the relative durations of successive developmental periods up to the beginning of gastrulation, with the following results (averages based on all 15 cases): extrusion of 2nd polar body till 1st cleavage: 3.27 units; onset of 1st till onset of 2nd cleavage: 1.22; cleavages 2–3: 1.08; cleavages 3–10: 1.00 each; cl. 10–11: 1.14; cl. 11–12: 1.24; cl. 12–13: 1.39; cl. 13–14: 1.68; cl. 14–15: 2.50; onset of 10th cleavage till first appearance of pigment concentrations (incipient gastrulation; around this time the fastest cleaving cells start their 15th cleavage): total duration 8.48.

The uncleaved eggs ranged in diameter from 1.8 to 2.3 mm (corresponding to a variation in volume of about 2×). Nevertheless all eggs showed essentially the same temporal cleavage pattern.

1b. Dissociation and cell counting

The study involving the counting of cells in dissociated and fixed blastulae (see previous report, sect. I.A.1b) was extended to obtain enough data for statistical treatment. The new data corroborate those given last year.

2. *Further study of embryonic axis formation by induced mesoderm in reaggregates (Ambystoma mexicanum, Triturus alpestris)* (P. D. Nieuwkoop)

This work was a continuation of experiments described in the previous report (sect. I.A.2) in which recombinates were made of dis/reaggregated animal (ectodermal) and vegetative (endodermal) material from blastulae. As mentioned in the previous report, the greatest difficulty in such experiments is the long period required for reaggregation (12–24 hrs.). By the time the inductive interaction occurs in the reaggregate, the ectodermal material has largely lost its competence for mesoderm induction, and the endodermal material most of its mesoderm-inducing capacity. Therefore, dis/reaggregation must be carried out at the earliest possible stage. However, the younger the embryo, the more susceptible are its cells to the damaging effect of the dis/reaggregation procedure.

It was tried in vain to reduce the harmful effect of the procedure by, among other things, adding serum globulin to the medium. The negative results may have been due at least partially to the relatively low vitality of the eggs available, a factor of particular importance when such high demands are put on the material.

As a control for the dis/reaggregation experiments it was necessary to check whether regional differentiation tendencies are present in the endoderm already at the middle blastula stage (stage 8½). To this end preliminary experiments were carried out in which intact presumptive

ventro-caudal endoderm was recombined with disaggregated cranial neural crest material and, conversely, intact dorso-cranial endoderm with disaggregated caudal lateral plate mesoderm. The aim was to see whether or not the regional quality of the endoderm would be changed by that of the (ecto)mesoderm. The results are being analysed.

3. *The induction of gastrulation (Ambystoma mexicanum)* (M. H. M. Doucet-de Bruïne – doctoral thesis project, Univ. of Utrecht – with financial support of the Netherlands Organization for the Advancement of Pure Research)

The data obtained in the past years are being prepared for publication. There have been no essential changes in the results as presented in the previous report (sect. I.A.3).

4. *The induction of primordial germ cells in urodeles (Ambystoma mexicanum)* (A. Sutasurja)

It is known that in urodeles the primordial germ cells (PGCs) are of mesodermal origin: they arise from the ventro-lateral mesoderm of the gastrula (Nieuwkoop, 1947). Recent recombination experiments involving ectoderm and endoderm of axolotl blastulae (see report of 1970, sect. I.a.3) have demonstrated the capacity of the endoderm to induce PGCs and blood cells in blastula animal "halves" along with other mesoderm (Nieuwkoop and Boterenbrood, in preparation). The aim of the present experiments was to study the competence of the ectoderm for the induction of PGCs by the endoderm.

4a. Experimental morphology

Axolotl blastulae of stage $8\frac{1}{2}$ were used. The animal ectodermal cap was isolated, excluding any already induced mesoderm, and then subdivided into four different zones: central, intermediate, dorsal peripheral, and ventral peripheral. Each of these zones was then recombined with the ventro-lateral portion of the endodermal yolk mass of a blastula of the same stage. The recombinates were reared for 21 days to insure full differentiation of the induced PGCs. Their numbers were counted in serial sections. The relative volumes and total cell numbers of the ectodermal zones used for recombination were determined in control blastulae.

The preliminary results show that PGCs are found in all types of recombinate. They occur together with other ventro-caudal structures such as blood cells, Wolffian duct, proctodaeum and intestine, and are usually located in or between endothelia. It may be concluded that PGCs can be formed from any region of the animal ectodermal cap. There is no statistical difference in number of induced PGCs between recombinates involving the central, intermediate, or dorso-peripheral zones of the ectoderm, but the number induced in the ventral-peripheral zone is much higher. The latter difference cannot be accounted for solely by differences

in volume and total cell number between the ectodermal zones used: these differences, though real, are relatively much smaller than the difference in PGC number. Neither is there a relation with the total mass of induced mesodermal tissue, this being on an average approximately equal in all recombinates.

In conclusion, the ventral-peripheral zone of the ectoderm, if brought under the inductive influence of the ventro-lateral endoderm, furnishes considerably more PGCs than the other zones of the ectodermal cap.

4b. Autoradiography (in co-operation with G. A. Ubbels)

In order to unequivocally prove the ectodermal origin (via the mesoderm) of the PGCs an autoradiographic cell labelling technique was used. ^{3}H-thymidine was carefully injected into the blastocoel of blastulae at stage 8, which were then fixed at $\frac{1}{2}$ hr. intervals. All nuclei were sufficiently labelled after 3 hrs.

Experiments have been started to test whether PGCs are still properly labelled 10 days after injection of ^{3}H-thymidine into the blastocoel at stage 8. If so, recombinations will be carried out involving labelled ectoderm and unlabelled endoderm, and conversely.

B. ELECTRON MICROSCOPY

1. *Cortical wound healing in amphibian eggs, an exploratory investigation (Xenopus laevis, Pleurodeles Waltlii)* (J. G. Bluemink)

The fine-structural reorganization in the egg cortex during wound healing was followed by means of transmission electron microscopy (TEM). The alterations observed were interpreted as manifestations of membrane growth, active or passive contraction of filament arrays, and cytoplasmic coagulation. In the published paper (see sect. VII, ref. 2) Holtfreter's concept of a "surface coat" and his interpretation of cortical wound healing was discussed. The published data constitute the starting material for further hypothesis formulation and testing.

2. *Critical-point drying of eggs and embryos (Xenopus laevis)* (J. G. Bluemink)

Surface alterations observed during egg cleavage, cortical wound healing, and closure of the neural tube have made it necessary to know where, when, and on which scale the cell surface changes. Scanning electron microscopy (SEM) offers the possibility to obtain additional information on the dimensions of the surface changes. An arrangement has been made with Dr. P. F. Elbers to use the SEM facilities available at the "Centrum voor Submicroscopisch Onderzoek van Biologische Objecten", University of Utrecht. Making use of the critical-point drying technique a method has been worked out to adequately preserve the three-dimensional shape of amphibian eggs and embryos for SEM analysis.

3. *The effect of cytochalasin B (CCB) injected at the onset of cleavage-furrow formation (Xenopus laevis)* (S. W. de Laat, D. Luchtel, J. G. Bluemink)

Electrical measurements of membrane resistance and membrane potential during cleavage, as well as TEM analysis, have shown that the cell surface along the furrow changes at about 7 mins. after the onset of cleavage. As a result the permeation conditions for externally applied substances may also change. It is conceivable that the observed delayed effect on cleavage of externally applied CCB (see previous report, refs. 1 and 2) depends on such a change. To test this possibility CCB was injected directly underneath the furrow at the very onset of cleavage.

For changes in gross morphology and electrical measurements, see sect. I.E.1 below (S. W. de Laat). The material for TEM analysis is now under investigation. Preliminary observations show that within 2 mins. of CCB injection the organized layer of parallel 80 Å filaments along the furrow bottom is dismantled, although intact individual filaments are still found along the periphery of the injected area. At the site of injection, instead of intact filaments masses of dense material not seen before are present. The possibility is not excluded that the disappearance of the filaments is due to the displacement of filaments from the region of injection peripherally. At any rate, the experiment has shown a short-term effect of injected CCB on the cell-contractile machinery. The primary site of CCB binding is still unknown.

4. *Local growth in membrane area during egg cleavage (Xenopus laevis)* (J. G. Bluemink)

Surface marking experiments using iron-oxide particles have provided evidence that during egg cleavage new membrane is added to the pre-existing cell surface. Normal and cytochalasin B (CCB)-treated eggs without vitelline membrane were used to demonstrate and rate new membrane formation directly.

In co-operation with Ir. J. J. Bezem (University of Utrecht) a method has been elaborated to measure certain parameters in uncleaved eggs from which the pre-existing geometrical egg surface area can be computed. It was found that the normal increase in surface area is not enhanced when the eggs are exposed to CCB up to 45 mins. *before* cleavage, but that it is significantly enhanced upon exposure to CCB *during* cleavage. Recent literature regarding CCB action suggests two alternative interpretations: either CCB interferes directly at the membrane level with the process(es) regulating membrane growth, or it interferes with a filament system subjacent to the cell membrane which controls surface rigidity. The yolk-laden egg has a tendency to flatten under gravity and to increase its surface/volume ratio. If CCB damages the cortical filament system, this may result in a decrease in cell surface rigidity. Thus, membrane

growth in the region of the furrow could be promoted by the tendency towards flattening and increase of surface/volume ratio.

5. *Ultrastructural changes accompanying membrane growth during cleavage, as visualised by ruthenium red staining for TEM and SEM analysis (Xenopus laevis)* (J. G. Bluemink)

Using a fixation fluid containing ruthenium red (RR), it could be demonstrated that about 7 mins. after the onset of cleavage a RR-positive cell coat has been formed exclusively along the furrow surface. Such a coat is not yet present 3 to 4 mins. after the onset of cleavage. Concomitantly with the appearance of the coat, cisternae of a cytoplasmic membrane system are found to be continuous with the cell surface (serial sections). These cisternae are RR-positive on their inner sides. Towards the egg exterior the staining is as intense as at the outer egg surface, whereas more internally the staining becomes progressively less intense.

Also at 7 mins. after the onset of cleavage, concentrations of RR-coated material showing myelin-like configuration are found along the furrow surface. In SEM micrographs they show up as spheres projecting from the furrow surface, which are still absent 3 to 4 mins. after the onset of cleavage. TEM-analysis reveals that similar material in the form of electron-dense droplets and plaques is present in the cytoplasm along the furrow. In CCB-treated eggs the morphological changes are not different from those in normal eggs. These morphological changes are correlated in time with changes in electrical potential and electrical resistance of the cell membrane (see sect. I.E.1), and are interpreted as manifestations accompanying membrane growth.

6. *Light and electron microscopy of cortical structures in the superficial and deep ectoderm of the embryo (Xenopus laevis)* (R. A. F. Dekker – doctoral thesis project, Univ. of Utrecht – with financial support of the Netherlands Organization for the Advancement of Pure Research)

Phase-contrast photomicrographs of $1\frac{1}{2}$ μ sections of whole blastulae, embedded in epoxy resin, show that by stage 9 (Nieuwkoop and Faber) the conditions for separation and isolation of the superficial (epithelial) and deep (neural and sensory) layers of presumptive ectoderm are optimal. The first purpose of this study was to compare the fine structure of the superficial and deep layers of the presumptive ectoderm. Some of the components of the cortical cytoplasm, particularly microfilaments and small electron-translucent vesicles, are delicate. Phospholipids and glycogen also are easily lost during the preparative procedure. Since it is thought that these components may play an important role in the physiological changes that occur after isolation of the deep ectoderm (see previous report, sect. I.B.3) an optimal fixation must be achieved.

A variety of conventional and non-conventional fixatives were tested. An adequate fixation procedure for presumptive ectodermal cells in the

blastula has not been found. Moreover, it was found that in cells of the superficial ectoderm the cortical layer is much less conspicuous than in uncleaved eggs. This suggests that ultrastructural differences between cortical areas in superficial and deep ectoderm are less marked than was expected, and that changes in fine structure, e.g. those resulting from isolation, will be difficult to analyse. For these reasons the original project has been abandoned. Since neurulae yield much better ultrastructural images this study is being continued with cells of the neural ectoderm (see 7 below).

7. *Ultrastructural studies on the closure of the neural tube (Xenopus laevis)* (R. A. F. Dekker – doctoral thesis project, Univ. of Utrecht – with financial support of the Netherlands Organization for the Advancement of Pure Research)

During neurulation the outer cell surfaces of the neural ectoderm acquire adhesive properties. These cells play an important role in the closure of the neural tube and in the reconstruction of the integrity of the overlying epidermis. Closure requires some of the neural ectoderm cells to adhere to each other. As a result, part of the cell surfaces which were originally external now become located more deeply. An ultrastructural study of the cortical cytoplasm of cells of the neural ectoderm involved in the closure of the neural tube may therefore provide information on (1) the mechanism of neural tube closure itself, and (2) the relation between fine structure and properties of outer and inner cell surfaces (cf. original project, 6 above).

7a. Transmission electron microscopy (TEM)

A variety of fixation procedures for neurulae of *Xenopus laevis* were tested (see Schroeder, 1970, Karfunkel, 1971, Burnside, 1971, and modifications of Kalt & Tandler, 1971). The original fixative of Kalt & Tandler resulted in approximately 20% shrinkage. Present modifications concern the buffer system, the composition and concentration of aldehydes and DMSO in the fixative, and the concentrations of added ions.

The present fixation experiments have led to the conclusion that the osmolarity of a fixative is not the only factor affecting swelling or shrinking of embryos. In fact preservation without swelling or shrinkage was achieved with fixatives of a wide osmolarity range (140–1400 mOsm). In this respect a very significant difference between cacodylate, phosphate, and s-collidine buffers was observed. The duration of aldehyde fixation clearly affects the amount of glycogen particles preserved. In general a short fixation time leads to less extraction of glycogen. Better results are also obtained if after aldehyde prefixation the embryos are directly transferred into ice-cold osmium tetroxide fixative.

Extracellular material can be visualized at the surface of neural ectoderm cells with ruthenium red. Preliminary results show that the presence and

ultrastructure of this material is stage-dependent. Experiments with purified ruthenium red added to the osmium tetroxide fixative are in progress.

7b. Further improvements of the fixation procedure for neurulae (in co-operation with J. G. Bluemink)

Our past experience has shown the difficulty of preventing loss of glycogen during fixation by changing the composition and concentration of the aldehyde fixative alone. Therefore other steps in the procedure have been modified as well. Modifications for optimal preservation of fine structure include: (1) stabilization of neurulae (without vitelline membrane) for 15 minutes in 1% distilled acrolein; (2) prefixation for only 2 hours (1 hour at room temperature, 1 hour on ice) in aldehyde fixative; (3) fixation in osmium tetroxide fixative (containing 1% $K_3Fe(CN)_6$) at 4° C, without rinsing between times; (4) dehydration in cellosolve at pH 12.0 for the lower concentrations.

7c. Scanning electron microscopy (SEM)

Neurulae (stages 12½–19) were fixed and prepared for examination in a Cambridge Stereoscan – S4. (The SEM facilities were made available by the "Centrum voor Submicroscopisch Onderzoek van Biologische Objecten", University of Utrecht, Director Dr. P. F. Elbers.) At the neural plate stage three areas differing in cell surface characteristics can be distinguished: (1) cells outside the neural plate have a rather smooth surface; (2) neural plate cells have many protrusions, probably microvilli; (3) cells at the periphery of the plate carry the largest number of protrusions. As the outer surface area of the cells decreases more protrusions per unit area become apparent. This may represent constriction of cell surfaces, possibly due to contractile action of microfilaments. Older neural-groove stages exhibit larger numbers of cells with microvilli on their surfaces.

7d. The effect of concanavalin-A on neurulation

The plant lectin, concanavalin-A (con-A), affects the normal development of neurulae inside their vitelline membrane. Treatment of neurulae (stages 13 through 18) with con-A (10–100 μg/ml culture medium) for 1–60 mins. generally results in abnormalities or inhibition of neural tube closure. Surprisingly, if the vitelline membrane is removed before treatment little effect on the process of closure is observed.

It is known that binding-sites for con-A can be exposed by treatment with proteases or urea. Therefore, prior to incubation of neurulae (without vitelline membranes) in a con-A-containing medium, a mild treatment with solutions containing trypsin or urea will be tried.

C. HISTO- AND CYTOCHEMISTRY

1. *Cytochemistry of the egg and early cleavage stages*

1a. Cytoplasmic differentiation near the bottom of the first cleavage furrow *(Ambystoma mexicanum)* (G. A. Ubbels)

As mentioned in the previous report (sect. I.C.1a) in freeze-dried eggs a glycogen- and pigment-rich region of cytoplasm, devoid of yolk granules, is present immediately around the bottom of the cleavage furrow. This region also contains numerous tiny ($\leq 1\ \mu$) granules staining for protein. It was suggested that this concentration of glycogen and protein-containing granules is somehow connected with the cleavage process, and that the latter granules might represent either mitochondria, lysosomes, or residual bodies of yolk granules.

This year the cytochemical characterization of the small granules was continued. They are never observed when using common histological fixatives followed by routine alcohol dehydration. In that case only a circumscribed "empty" region is seen at the base of the furrow. The small granules occur consistently in eggs prepared by freeze-substitution, using either Gendre or absolute alcohol as the substitution fluid, or by freeze-drying. They stain with Hori's modified acid hematein test and with Chang's aniline acid fuchsin, which are both conventional methods for demonstrating mitochondria. However, yolk platelets also stain with these methods, so that a clear-cut discrimination between the small granules and the smallest yolk platelets is impossible. Moreover, both mitochondria and lysosomes contain phospholipid and consequently the latter also react with the acid hematein method.

Similar small granules are also observed in the region of the diastema, where the new cell membrane will appear.

At present the distribution of mitochondria and lysosomes throughout the egg is being studied. For the former we will use cytochemical methods to demonstrate tetrazolium reductase and succinic dehydrogenase, for the latter an acid phosphatase reaction and a non-enzymatic fluorescence reaction using acridine orange. In addition, tests for some other hydrolytic enzymes may provide information on metabolic and synthetic processes going on in the egg during first cleavage, particularly in the neighbourhood of the cleavage furrow. The critical application of such tests to the yolk-rich axolotl egg is not very easy, due mainly to the difficulties inherent in the production and post-treatment of frozen sections. The use of paraffin wax of low melting-point considerably diminishes these problems.

1b. Cytoplasmic differentiation in the subcortical region between oviposition and first cleavage *(Xenopus laevis)* (G. A. Ubbels, R. T. M. Hengst)

In the axolotl egg small yolk-free, glycogen-rich regions appear in the animal hemisphere immediately after oviposition. They ultimately fuse

to form a subcortical band of yolk-free cytoplasm in the future furrow region (see previous report, sect. I.C.1a). If, as suggested, this cytoplasmic segregation is connected with furrow formation, it may be expected to occur also in other amphibian species. Therefore, cytoplasmic differentiation in this region is being investigated in *Xenopus*, and a cytochemical analysis has been started.

1c. Cytoplasmic differentiation during grey crescent formation *(Discoglossus pictus)* (J. Klag, G. A. Ubbels)

Observations made by Ancel and Vintemberger in *Rana fusca*, and later by Glade in *Xenopus laevis* (unpublished work performed at the Hubrecht Laboratory – see report of 1969, sect. I.C.3) suggest a displacement of cytoplasmic constituents towards the future dorsal side in newly fertilized eggs. In *Xenopus* various cytoplasmic constituents such as RNA, glycogen, ribosomes, and mitochondria accumulate on the vegetative side of the nucleus prior to nuclear breakdown (Brachet *et al.*, 1970). They could subsequently be displaced towards the future grey crescent region by the suggested cytoplasmic streaming movements, thus playing a part in the formation of the grey crescent, and thereby in the establishment of polarity in the future endoderm (according to Nieuwkoop's findings the latter is responsible for the dorso-ventral polarization of the embryo during mesoderm induction in the blastula).

To study this problem further, a species was chosen in which grey cresent formation is rather pronounced: *Discoglossus pictus*. Cytochemical differentiation is being studied in eggs from oviposition till first cleavage. The preliminary results indicate that the suggested cytoplasmic streaming movements do indeed take place also in *Discoglossus*. The investigation is being continued in Poland.

2. The project mentioned in section I.C.2 of the previous report has been discontinued. The work of A. Sutasurja is described in section I A.4 above.

D. BIOCHEMISTRY

1. *Patterns of soluble proteins in early development (Ambystoma mexicanum)* (P. Th. M. van der Saag; S. K. Brahma, Lab. of Med. Anat. and Embryol., Univ. of Utrecht).

Most studies on protein patterns during early amphibian development suffer from a number of inconsistencies. First of all, most authors are dealing with a very limited number of protein bands obtained by subjecting the soluble protein fraction to polyacrylamide gel electrophoresis. Secondly, one has so far only compared the staining patterns of the proteins present and not their synthesis, which requires studying the incorporation of radioactive amino acids, an approach hampered by the well-known impermeability of the amphibian embryo to many compounds.

We have used the technique of dissociation and reassociation combined with radioactive labelling described in detail in ref. 4. Fractionation of the soluble proteins of axolotl gastrulae in this way yielded more than 20 stained bands, which is more than other authors have obtained so far. Autoradiography of the gel showed that not all stained bands were labelled, and that some were more strongly radioactive than others.

Preliminary experiments on various other embryonic stages have indicated that although no changes are detectable in the staining pattern between unfertilized eggs and the tailbud stage, the pattern of protein synthesis (labelling pattern) shows marked changes from stage to stage.

Experiments are in progress in which the radioactivity patterns obtained by the dissociation procedure are compared with those obtained by the micro-injection technique described in section I.E.4 below.

2. *Protein synthesis in the neonatal brain (Mus musculus).* See section IV.C., page 51.

E. BIOPHYSICS

1. *The action of cytochalasin B (CCB) during egg cleavage: dependence on cell membrane permeability (Xenopus laevis)* (S. W. de Laat, D. Luchtel, J. G. Bluemink)

This work is a continuation of that described in the previous report (sect. I.E.2). By exposing eggs during first cleavage to CCB for successive periods of 4 mins., it has been shown that CCB sensitivity, resulting primarily in furrow regression and exposure of newly-formed membrane to the outer medium, and secondarily in abnormal development, becomes manifest approximately 7 mins. after the onset of first cleavage. This may indicate that CCB is unable to penetrate before that time. To eliminate the effect of a possible permeability barrier and to test the CCB sensitivity of the operative contractile system, CCB was injected just beneath the cell membrane in the furrow (cf. 4 below). The test solution contained 10 μg CCB and 1% dimethylsulfoxide (DMSO) per ml Steinberg solution. The quantity injected was 0.02 μl. After the injection microphotographs were made at short intervals.

When CCB is injected immediately beneath the membrane in the middle of the furrow the shallow groove begins to regress within a minute at the site of injection, while the process of cleavage continues on either side of the injection area. Control eggs were injected with 0.02 μl Steinberg solution with or without 1% DMSO. No furrow regression results from such injections.

As an additional control it was tested whether or not the presence of divalent cations influenced the immediate effect seen after the injection of CCB. Eggs were placed in Steinberg solution prepared without the addition of Ca^{++} and Mg^{++}, and injected with 0.02 μl of a solution con-

taining 10 μg CCB/ml and 1% DMSO in Steinberg solution without divalent cations. The short-term effect was no different from that seen in the main CCB-injection experiments.

When CCB is injected immediately beneath the cell membrane just in front of one of the advancing furrow ends, the cleavage furrow stops at the injection site. The other end of the furrow continues to advance in the opposite direction, proceeding around the vegetative pole. When DMSO is injected in front of one of the advancing furrow ends the furrow proceeds through the injected area.

From these experiments it was concluded that the contractile system is sensitive to CCB and that a permeability barrier prevents externally applied CCB from being effective before 7 mins. after the onset of first cleavage. To obtain further evidence for a change in membrane permeability properties the membrane potential (Vm) and membrane resistance (Rm) were measured continuously in normally cleaving eggs and in cleaving eggs injected with CCB, using micro-electrode techniques. During normal cleavage Rm remains relatively constant for about the first 7 mins. of cleavage and then decreases, reaching a lower plateau about 10 mins. later. Vm shows a pattern inverse to that of Rm. When CCB is injected immediately after the onset of first cleavage, first Vm changes slightly and Rm drastically during the injection procedure due to the insertion and withdrawal of the micro-injection pipette. However, after wound closure Vm and Rm show patterns similar to those of an uninjected egg, although the furrow regresses at the injection site.

It was concluded (1) that there is a relationship between sensitivity to externally applied CCB and changes in membrane permeability properties during cell division, and (2) that CCB has no effect on the electrical membrane properties. The results are in press (see ref. 21; also ref. 8).

2. *Ion activities and ion permeability properties during pregastrula development (Xenopus laevis)* (S. W. de Laat, R. J. A. Buwalda)

This work is a continuation of that described in the previous report (sect. I.E.3). Sodium-selective glass micro-electrodes and potassium- and chloride-selective liquid ion-exchange micro-electrodes with excellent stabilities and selectivity properties have been developed.

Simultaneous measurements of sodium, potassium, and chloride activities and membrane potential and resistance have been carried out during first cleavage. The sodium activity in the uncleaved egg is about 20 mM. This means that 60–70% of the total sodium content is present in bound form. During cleavage the sodium activity increases by about 2 mM. The potassium activity is about 50 mM at the onset of cleavage. Probably all potassium ions are osmotically active. During cleavage the potassium activity decreases by about 2 mM. The chloride activity in the uncleaved egg is about 55 mM and decreases by about 2 mM during cleavage. None of the changes in ion activity can account by itself for the observed

change in membrane potential from about −10 mV to about −20 mV during cleavage.

Similar measurements combined with alterations of the ionic composition of the medium indicate that the change in membrane potential is probably due to an increase in potassium permeability. This increase can be correlated with the insertion of new membrane material with a relatively high potassium permeability into the outer surface (see 1 above).

3. *Electrical properties of membranes of pregastrula embryos (Xenopus laevis)* (S. W. de Laat, P. W. J. A. Barts).

This work is a continuation of that described in the previous report (sect. I.E.1). Using an automatic data acquisition system, detailed measurements were made of the changes in membrane potential and input and transfer resistance during early cleavage stages. The membrane potential shows cyclic changes corresponding to the cell cycle and increases from about −10 mV at the uncleaved stage to about −40 mV at late cleavage stages. The input resistance shows an inverse pattern. Transfer resistances are relatively low until the 16-cell stage, when apparently some intercellular membranes become impermeable to ions. Work is in progress to determine whether it is the existing membranes that become impermeable or whether newly formed membranes have permeability properties different from those already present.

4. *Method for intracellular injection of nanoliter quantities* (S. W. de Laat, P. Th. M. van der Saag).

In amphibian embryos drug effects and the incorporation of radioactive isotopes often cannot be studied in the usual way, i.e. by incubation in an appropriate medium, since these molecules do not enter the cytoplasm in sufficient quantities. Thus it became necessary to develop a microinjection device that allows for multiple injections of quantities between 10 and 100 nl (the amphibian embryo having a volume of about 1–5 μl) with sufficient reproducibility and accuracy.

The device consists of a commercially available microliter syringe. The plunger is connected to a micrometer with a non-rotating axis. A glass micro-capillary is pulled on a micro-electrode puller (tip diameter 2–10 μ) and mounted on the syringe. The system is filled with water. The fluid to be injected is separated from the water column by a drop of paraffin oil. The quantity injected can be read off from the micrometer scale. The eggs to be injected are placed in small holes in a turntable that turns underneath the micro-capillary. This allows rapid injection of large numbers of eggs. The reproducibility and accuracy were checked by injecting ^{3}H-leucine both into water and into *Xenopus* eggs. The system is linear at least between 10 and 80 nl and has a reproducibility of about 5%. The details will be published.

II. Amphibian development (general)

1. *Relation between size and regional differentiation of neural tissue masses (Triturus alpestris)* (E. C. Boterenbrood)

Both in reaggregates (Boterenbrood, Ph.D. thesis, 1962) and in transplants (see previous report, sect. II.1) of neural cell material originating from the anterior neural plate region, the differentiation of telencephalic, dorsal diencephalic, and dorsal mesencephalic structures seems to be favoured in smaller cell masses. This suggests that the regional differentiation of specific brain parts is related to "peripheral conditions" obtaining in the cell mass, and that such conditions also play a role in the establishment of the pattern of presumptive regions in the normal neural plate.

A systematic investigation into the possible relations between size and regional differentiation of neural cell masses has been started. In preliminary experiments a given area of the neural plate of fixed dimensions was taken as a "unit". This area was excised and dis- and reaggregated either alone or in combination with similar units from other donor embryos, to obtain spherical cell masses of increasing size. These were then transplanted to the ventro-lateral mesoderm-free area of host neurulae to study their differentiation. The area chosen as a unit was the median part of the anterior neural fold, which at stage $14\frac{1}{2}$ possesses differentiation tendencies for telencephalic, ocular, and diencephalic structures. No results are available yet.

III. Avian development

A. EXPERIMENTAL MORPHOLOGY

During the International Research Group period two members were introduced by K. Hara into the methods of avian experimental embryology. Subsequently the following research projects were started:

1. *Establishment of the medio-lateral pattern of differentiation in the neural plate (Gallus domesticus)* (M. K. Khare)

The establishment of the pattern of neural differentiation along the antero-posterior axis of the neural plate is strictly related to, and most probably determined by, the development of the underlying mesodermal substrate (Hara, Rao). About the formation of the pattern in the medio-lateral (=future ventro-dorsal) direction very little is known, however.

In a first approximation to this problem, pieces of ectoderm were isolated from the level of the presumptive mesencephalon, and their self-differentiation studied by means of the intra-coelomic grafting technique. The stages ranged from the definitive primitive-streak stage to the long head-process stage. The ectodermal pieces were taken both from the median region overlying the head-process and from the adjacent lateral regions.

The preliminary results show that differentiation tendencies for dorsal structures (optic tectum) first appear in the median region (youngest stage used) and only shift towards the lateral regions in later stages. The investigation is being continued in India.

2. *Differentiation tendencies of Hensen's node (Gallus domesticus)* (M. Veïni)

Hensen's node in avian embryos is considered as being homologous to the amphibian dorsal blastoporal lip. Recent autoradiographic studies have shown that prospective endodermal cells contained in the node finish their ingression and form the definitive endoderm by the definitive primitive-streak stage. After this stage the node contains only prospective mesodermal cells. However, there still is a paucity of information concerning the exact differentiation tendencies of the node around the definitive primitive-streak stage.

To fill this gap the following experiments were performed. Hensen's node was isolated in one piece from blastoderms of middle-streak to head-fold stages, from which the endodermal layer had been previously removed. The isolates were then cultured *in vivo* by means of the intracoelomic grafting technique.

The preliminary results show that endodermal differentiation tendencies had almost completely disappeared after the definitive primitive-streak stage, whereas the control isolates (nodes with the endodermal layer intact) gave rise to endodermal structures throughout the stages used. Neural differentiation tendencies were always found in nodes isolated from the definitive primitive-streak stage onwards, but in nodes isolated from younger stages the frequency of neural differentiation was significantly lower. The investigation is being continued in Greece.

B. ORGAN CULTURE

1. *Proportionate growth of the embryonic appendicular skeleton (Gallus domesticus)* (J. M. van Lenthe)

It has been suggested that one of the factors contributing to the faster growth of the metatarsus compared with the radius is a higher matrix-synthesising capacity of the metatarsal cells, reflected in a higher rate of incorporation of $^{35}SO_4$/DNA *in vitro* (see K. A. Lawson, previous reports). Quantitative autoradiography of incorporated $^{35}SO_4$ has been used to determine whether this difference is localized in particular regions of the rudiment.

Glutaraldehyde fixation was used since liquid scintillation counting showed that rudiments so fixed contained the same total amount of incorporated $^{35}SO_4$/DNA as rudiments prepared by the method used for the earlier biochemical experiments.

Since the accuracy of grain counting in ^{35}S autoradiographs is influenced

by the thickness of both section and emulsion layer, the method has been made as precise as possible for light microscopy by using 1 μ sections of material embedded in Epon; acceptable limits for reproducibly thin emulsion layers have been established on test slides using the dipping technique and Kodak NTB_2 emulsion.

Preliminary inspection of the labelled rudiments shows greatest incorporation to be in the young hypertrophic zone, somewhat less in the flattened cell zone, and little in the mature hypertrophic zone and epiphyses. The detailed analysis between rudiments is not yet complete.

2. *Tissue interactions in organogenesis*

Three members of the International Research Group worked in association with K. A. Lawson on problems connected partially or entirely with tissue interactions in organogenesis. One of the projects is described below (Navagiri), the two others (Nessi, Suhana) will be found in sections IV.A.3 and IV.B.1.

2a. Perichondral ossification in long-bone rudiments *(Gallus domesticus)* (S. Navagiri)

Perichondral ossification begins in association with hypertrophic cartilage. Cartilage which does not hypertrophy does not ossify perichondrally. The possibility of an inductive relationship between hypertrophic cartilage and the transformation of the perichondrium to an osteoid-producing periosteum is being investigated *in vitro*.

Initial experiments were designed to establish the conditions under which viable long-bone cartilage could be obtained completely free of perichondral cells. Seven to eight-day old tibiae were dissected to yield an area from the proximal half consisting of a perichondral sleeve around a small part of the epiphysis, the complete flattened cell zone, and young hypertrophic cartilage. The perichondrium was removed with various techniques: critical examination of the cartilage before, during and after culture revealed that only mechanical stripping followed by a few minutes' treatment with proteolytic enzymes is a promising method for complete removal of perichondral cells. The naked cartilage grew in length during 6 days culture, but at a slower rate than the intact fragments. In addition, the characteristic organization of flattened and hypertrophic cells was lost in the absence of the perichondrium, in contrast to the fragments with perichondrium where normal chondrogenesis was maintained and osteogenesis initiated. Isolated perichondrium formed nodules of cartilage and, in some instances, separate patches of osteoid tissue.

The results may be due to 1) the dissociation technique, 2) the provision by the connective tissue of a suitable micro-environment for normal cartilage development, 3) a localized contribution of new cartilage cells by the perichondrium. All possibilities can be experimentally tested. The investigation is being continued in India.

IV. Mammalian development

A. TISSUE INTERACTIONS IN ORGANOGENESIS

(See introduction to section III.B.2.)

1. *Mesenchyme specificity in salivary gland development (Rattus norvegicus, Mus musculus)* (K. A. Lawson)

The salivary system is frequently quoted as illustrating high specificity in mesenchymal requirement by the developing epithelium on the basis of Grobstein's and Wessell's work, in which mouse submandibular epithelium showed morphogenetic activity only when associated with its own mesenchyme *in vitro* and not in the presence of e.g. lung mesenchyme. In contrast, rat parotid epithelium is able to develop morphogenetically and functionally not only in salivary but also in lung mesenchyme (see ref. 9).

The mesenchyme requirement of both salivary glands in the two species has been reinvestigated, particularly with respect to the ability of lung mesenchyme to support development of the epithelium. The epithelial bud of both glands in both species is able to undergo morphogenesis and cytodifferentiation in lung mesenchyme. The recombinate of mouse submandibular epithelium with mouse lung mesenchyme remains small and loses cells after 3 days *in vitro*, in contrast to the homotypic submandibular and lung control recombinates; subsequent experiments showed that the morphogenesis of mouse submandibular epithelium in mouse lung mesenchyme is dependent on the quantity of lung mesenchyme initially provided. In contrast, recombinates of mouse submandibular epithelium with rat lung mesenchyme do not lose cells, and preliminary experiments suggest that submandibular mesenchyme and rat lung mesenchyme are quantitatively equivalent for supporting salivary morphogenesis.

Histological examination of early recombinates indicates that mouse lung mesenchyme is more sensitive to handling damage than rat lung mesenchyme, but this observation cannot explain the failure of small quantities of mouse lung mesenchyme to maintain salivary morphogenesis after the first 3 days *in vitro*.

These results support the suggestion made in the previous report (sect. IV.A.1) that the mesenchymal requirement of both rat and mouse salivary systems is less specific than was previously supposed.

2. *Cytodifferentiation of salivary epithelium in lung mesenchyme (Rattus norvegicus, Mus musculus)* (K. A. Lawson)

In addition to the normal adenomeres formed by rat parotid epithelium in lung mesenchyme, 40% of the recombinates also contain masses of tubules with much PAS-positive material (see ref. 9). Since the presence of glycogen in mouse bronchial epithelium is associated with the morphogenetic activity of this epithelium and requires the presence of lung

mesenchyme, the possibility that lung mesenchyme is able to induce glycogen synthesis in salivary epithelium has been investigated in both rat and mouse salivary systems.

Striking quantities of glycogen are present in the tubular portion of salivary-lung recombinates in both species. Little or no glycogen is present in the same area in homotypic recombinates of parotid, whereas slight quantities were found in the rat submandibular, and substantial amounts in the mouse submandibular. In the absence of a reliable histochemical method for the determination of glycogen, further quantitative analysis has not been attempted.

These results suggest that lung mesenchyme can have a modulating influence on glycogen metabolism in salivary epithelium, but does not activate metabolic pathways not normally present.

3. *Erythropoiesis in fetal liver (Mus musculus)* (A. C. Nessi)

The liver is the major erythropoietic organ during the second half of mammalian prenatal development. The origin of the erythropoietic precursor cells, which can first be identified at the boundary between the endoderm and mesenchyme of the liver rudiment, is controversial, as is the subsequent control of the development of the erythroid series. A culture system in which the normal relationship between liver endoderm and mesenchyme can be maintained or altered in a controlled way would offer a basis for the analysis of the function of these two components in the initiation and control of erythropoiesis.

Hepatic rudiments, with and without additional splanchnic mesenchyme, from 24–31-somite mouse embryos were cultivated for 3 or 6 days in order to establish whether liver erythropoiesis can be initiated and maintained *in vitro*. Presumed erythroid cells with pycnotic nuclei were found in the mesenchyme of explants from younger embryos; explants from embryos of 27–31 somites had prominent areas of cells with small, densely basophilic nuclei. The complete erythroid series has not yet been found in these explants.

As a standard for the quantitative analysis of the experimental material a quantitative analysis of erythropoiesis in intact liver during normal development has been begun. Of the various fixatives tested – Helly's (formol-Zenker's), formol-acetic-Zenker's, Bouin's, Carnoy's, and perfusion with glutaraldehyde via the umbilical vein – only Helly's with subsequent staining of paraffin sections with Mayer's haemalum, benzidine (for hemoglobin) and Giemsa proved adequate for light microscopical study. The investigation is being continued in the Argentine.

4. *Interaction of interstitial cells and tubule epithelium during spermatogenesis (Rattus norvegicus)* (Suhana)

See section B.1 below.

B. SPERMATOGENESIS

1. *The effect of human chorionic gonadotrophin (HCG) on spermatogenesis (Rattus norvegicus)* (Suhana)

Four days after birth the epithelial tubules of the rat testis consist only of gonocytes and supporting cells. Fragments of such testes have been cultured in a chemically defined medium (Steinberger) in the presence of various concentrations of HCG (Organon).

The gonocytes in both control and hormone-treated explants developed into resting spermatocytes after one week of culture, but no further development of the germinal epithelium occurred. Degeneration of the germinal epithelium began during the fourth week in the control explants and in those treated with 25 i.u. HCG; this degeneration was advanced to the third week by 50 i.u. HCG, and to the second week by 100 and 200 i.u. HCG. That this was not a non-specific toxic effect was evident from a stimulating effect of HCG on the interstitial cells: stimulation increased with increasing HCG concentration.

A method was developed for dissociating tubules from interstitial cells using pronase and differential centrifugation. Preliminary results suggest that the gonocytes in isolated tubules do not develop into type-A spermatogonia after one week of culture, whereas they do in the tubules recombined with interstitial cells. The investigation is being continued in Indonesia.

C. BRAIN DEVELOPMENT

1. *Protein synthesis in the neonatal brain (Mus musculus)*

Biochemical research in this Laboratory in 1972 has placed emphasis on developing the necessary approaches for further exploration of the developing neonatal mouse brain as a suitable system for the study of molecular developmental biology, particularly in relation to the role of protein synthesis and its regulation during differentiation.

1a. *In vitro* protein synthesis on neonatal brain polyribosomes (P. Th. M. van der Saag, Th. F. J. de Greeff)

Free polyribosomes were isolated without using detergents, by centrifugation of a 10,000 × g supernatant through 2 M sucrose. Membrane-bound polyribosomes were isolated from the 10,000 × g sediment by the same method after detergent treatment. The total polyribosomal yield is 3 mg polyribosomes/ gm wet weight of tissue; 40–60% of this is in the membrane-bound state. Of these ribosomes 80–90% are present in structures heavier than dimers, as revealed by zonal centrifugation through sucrose. Under conditions suitable for cell-free protein synthesis the polyribosomes are very active, using their endogenous mRNA as a template; 200–300 amino acids/ribosome/ hour are incorporated, a level of incorporation comparable with that of the most active similar systems from other mammalian sources.

The system is characterized by a very low level of ribonuclease and linear incorporation for 40–60 mins. at 37° C. It was ascertained that the main activity of the system is to complete pre-existing nascent chains rather than to re-initiate new chains, because of a lack of initiation factors (see 1c below). For reasons so far unknown only about 30–50% of the chains are released from the ribosomes.

It has been found that the free polyribosomes are active in the completion of nascent chains of the micro(neuro-)tubule protein, tubulin, which is a protein of great abundance in this tissue.

1b. A reconstituted cell-free protein-synthesizing system employing purified ribosomes and ribosomal subunits from neonatal brain (P. Th. M. van der Saag, Th. F. J. de Greeff)

For the preparation of uncomplexed ribosomes and ribosomal subunits the method of Blobel and Sabatini (1971) has been followed, using purified polyribosomes or a 100,000 × g microsomal sediment as starting material. It was shown by several criteria that the ribosomes prepared with this method are free of messenger RNA, peptidyl-tRNA, and aminoacyl-tRNA. They were found to be very active with poly-U as an artificial messenger: 4–5 phenylalanine residues/ ribosome/min. were incorporated linearly for 15–20 mins. The product of this incorporation was isolated and subjected to paper chromatography using standard methods. The results show that only polyphenylalanine is synthesized, thus excluding the possibility of a single-step addition of phenylalanine to the peptidyl-tRNA still present on the ribosome when different methods of ribosome isolation are employed. At least 75% of the ribosomes are active in the cell-free system.

Ribosomal subunits were prepared by centrifugation of such purified ribosomes in high-salt gradients in a Beckman B XIV rotor in collaboration with Dr. B. A. M. van der Zeyst (Van 't Hoff Laboratory, University of Utrecht). Upon incubation in a poly-U-dependent cell-free system these subunits together were as active as the non-dissociated 80s ribosomes. This reconstituted system is suitable for use with other templates, preferably natural mRNAs, as a means of identifying their coding properties.

1c. The isolation and characterization of protein synthesis-initiation factors from neonatal brain (P. Th. M. van der Saag, Th. F. J. de Greeff)

For the preparation of crude initiation factors a 100,000 × g microsomal sediment was extracted with 0.5 M KCl according to the method of Anderson *et al.* The preparation obtained increases – at concentrations of 0.1–0.5 mg protein/ml – 5 to 10-fold both the rate and extent of either endogenous mRNA-directed polypeptide synthesis on polyribosomes *in vitro*, or poly-U-directed polyphenylalanine synthesis on purified ribosomes. Several criteria for the initiation-promoting character of this factor preparation were tested and found to be positive.

Preliminary experiments were performed involving the application of

the crude preparation to DEAE-cellulose columns and stepwise elution by KCl (0.05–0.5 M); it was found that most of the isolated fractions have stimulatory effects on *in vitro* protein synthesis, certain combinations of fractions showing more than additive effects.

1d. A ribosome dissociation factor from neonatal brain (P. Th. M. van der Saag, S. Bordin)

At least in bacterial systems it is well established that one of the initiation factors (IF3) also functions in promoting the dissociation of 70s monomers into 30s and 50s ribosomal subunits, a prerequisite for polypeptide chain initiation. In eukaryotic systems the existence of a dissociation factor functioning in the same step in the ribosome cycle has not so far been demonstrated in the same definitive way.

We found that a crude 0.5 M KCl extract of the 100,000 × g microsomal pellet of 5 to 7-day brain possesses ribosome dissociation activity. Purified ribosomes (see 1b. above) were used as a substrate and more than 60% dissociation could be obtained under optimal conditions. The dissociation reaction appears to be stoichiometric rather than catalytic and can be completely reversed by high Mg^{++} concentrations. After this factor-mediated dissociation the ribosomes are still very active in poly-U-dependent systems.

Upon DEAE-cellulose chromatography most of the dissociation activity elutes around 0.15 M KCl, as in *E. coli*. This more purified preparation showed the same properties as the crude extract but could be used in 5–10 times lower quantities to obtain the same effects.

Further investigations will be needed to assess the exact role of this factor in the process of polypeptide chain initiation.

1e. The isolation and characterization of messenger RNA from neonatal brain (P. Th. M. van der Saag, C. I. M. Bänziger)

Since neonatal mouse brain is such a rich source of the microtubule protein, tubulin, it seemed promising to study the mRNAs of this system generally and that for tubulin in particular. Neonatal mice were injected with ^{3}H-uridine or ^{3}H-adenosine and polyribosomes were isolated after 60 mins. labelling *in vivo*. This procedure leads to a preferential labelling of polysome-associated mRNA, while ribosomal RNA is only labelled to a small extent. It was shown that labelled RNA isolated from the polyribosomes behaves like mRNA in several respects: in particular, it was found that 70–80% contains poly-A sequences. Further, the RNA binds to nitrocellulose filters in 0.5 M KCl, and RNA in polyribosomes labelled with ^{3}H-adenosine is much more resistant to digestion with RNase A than that from polyribosomes labelled with ^{3}H-uridine (80% vs. 20%). Upon phenol extraction of polyribosomes at pH 9 the specific activity of the RNA is 3–5 times as high as that of RNA obtained at pH 7, while the former RNA shows a more heterogeneous sedimentation pattern than

the latter, which is primarily ribosomal RNA. When polysomal RNA is fractionated on sucrose gradients and individual fractions are treated either with RNase A and T_1 RNase, which cannot digest poly-A sequences, or with T_2 RNase, which destroys such sequences, some idea can be obtained about the size distribution of the mRNA present in the polyribosomes.

Beside these analytical techniques, preparative methods have been developed to isolate mRNA populations or individual species of mRNA using poly-A as a powerful tool. Our first results of chromatographing total polyribosomal mRNA preparations on cellulose-poly-U columns indicate that at least 70% of the labelled RNA can be bound to the column. This RNA can subsequently be eluted for further characterization.

Zonal centrifugation of polysomal RNA obtained by SDS-treatment of polyribosomes offers another possibility for obtaining large quantities of fractionated RNA. This method, in which up to 500 mg of polyribosomes can be used in a single run, is being applied in collaboration with Dr. B. A. M. van der Zeyst (Van 't Hoff Laboratory, University of Utrecht).

A beginning has been made with testing the RNA fractions obtained by these various methods for biological activity in either the reticulocyte lysate system, the *Xenopus* oocyte, or the fractionated brain system.

1f. Developmental aspects (P. Th. M. van der Saag)

One of the developmental problems in this system is the age-dependent decline of the level of protein synthesis in the maturing brain. Johnson *et al.* have extended this phenomenon, originally described for the *in vivo* situation, to a cell-free protein-synthesizing system, using both endogenous templates and poly-U. Particularly their poly-U-dependent system has been employed to demonstrate that ribosomes from 1 to 2-day mice are two to three times more active than those from 20-day or adult mice.

One of the aims of our studies has been to establish cell-free systems derived from brain tissue using components as well characterized as possible, and to exploit these systems for a developmental approach. At the same time the highest aims as to level of activity of the systems were set. Neither with our endogenous system, nor with the poly-U-dependent system with purified ribosomes have we been able to demonstrate a consistent difference in activity between ribosomes from brains of different ages. Microsomal systems programmed with either poly-U or endogenous templates were also tested and gave identical results. There are two possibilities to explain this discrepancy. First, the level of activity of our systems is about 50 to 200-fold higher than that of most of the brain systems used so far. Secondly, our purified ribosome system clearly uses better defined ribosomes than is common practice in other studies in this field.

It will be clear that a more detailed study will be necessary to specify the precise point(s) of action of this type of developmental regulation,

which has been characterized as a situation analogous but reverse to that in the sea urchin egg before and after fertilization. As in the latter case, the role of elongation and initiation factors, the percentage of ribosomes engaged in protein synthesis at a given developmental stage *in vivo* and *in vitro*, the role of transfer-RNA, the structure of the ribosome, and the roles of different ribosome populations (membrane-bound or free, neuronal or glial) are questions of high relevance but barely touched upon so far in systems displaying developmental regulation of protein synthesis at the translational level.

That the availability of identified homologous mRNA species would be of great importance in this respect needs no emphasis. The isolation of mRNA for tubulin would be a major step in this direction, particularly since this protein is so intimately connected with the differentiation of the neurones (outgrowth of neurites), both *in situ* and in isolated cell lines such as neuroblastoma cells.

V. Pattern formation in insects

During the past few years work on *Drosophila* in this Laboratory has undergone a shift in emphasis from genetical to morphogenetic aspects (see previous report, sect. VI.A). Genetic methods are now used primarily as a tool to affect pattern transformations in imaginal discs, and wild-type discs are increasingly studied to provide normal baselines for pattern formation.

1. *Determination, regulation, and positional information in imaginal discs (Drosophila melanogaster)*

1a. Duplication and regeneration in the *loboid-ophthalmoptera* eye disc (J. J. Holden, W. J. Ouweneel)

Mature *ld-opht* eye discs, in which the anlage for homoeotic wing tissue is located in the lateral half, were cut into 2/3 lateral and 1/3 medial fragments. These were cultured in adults for 8 to 28 days in order to allow the formation of a proliferation blastema at the wound surface, and then underwent metamorphosis in host larvae, to see whether either fragment was able to regenerate the missing structures (including homoeotic tissue) or to duplicate itself. Because of technical problems only a small number of differentiated implants have been obtained so far, which allow only very preliminary conclusions. Medial fragments seemed to be able to regenerate (in one case also wing tissue), but also produced unexpected duplications (particularly of the ocelli). The lateral fragments gave rise to some very significant duplications (particularly of the vibrissae).

1b. Duplication and regeneration in the wild-type haltere disc (W. J. Ouweneel, J. M. van der Meer)

Anterior and posterior fragments of mature haltere discs (cut at the $\frac{1}{2}$ or $\frac{3}{4}$ level) were cultured in adults and subsequently carried through metamorphosis in host larvae (cf. 1a. above; cf. previous report, sect.

VI.A.2b). (N.B. the anlagen in the organ map are mainly arranged *concentrically*, not sequentially as was suggested in the previous report – see ref. 11, 24.) Analysis of the metamorphosed implants showed that ¾ anterior (proximal) fragments formed blastemas from which posterior (distal) structures arose by regeneration. Some structures were regenerated in duplicate. It seems that the metathorax anlage is able to regenerate a complete haltere. This has to be checked, however, by transections at more proximal levels (in progress; results not yet analysed).

On the other hand, ¼ posterior (distal) disc fragments formed blastemas in which the distal structures normally produced by these fragments were duplicated in mirror image. This led to the formation of bilaterally symmetrical transplants. The posteriormost anlagen, i.e. those of the metathoracic bristle group, the capitellum, and the ventral pedicellar and scabellar sensilla groups, were duplicated most frequently. The strictly symmetrical structure of these transplants (as well as of the ½ posterior fragments, which are being analysed at present) suggests two important preliminary conclusions which have already been anticipated theoretically (see ref. 14): (1) if proliferation is limited, a small blastema is formed; nevertheless the fragment and blastema together seem to "split up" symmetrically ("homonomous arealization") and to produce only the most posterior anlagen in duplicate, while the more anterior anlagen originally present in the fragment drop out altogether; this would imply a partial "repatterning" within the original disc fragment; (2) if proliferation is very extensive, a blastema is formed that is larger than the original fragment; here also the fragment and blastema together seem to "split up" symmetrically, and now produce not only all the structures typical of the original fragment in duplicate, but also more proximal structures for which no anlagen were present in the original fragment (see ref. 15, 23).

VI. Miscellaneous

1. Dr. W. P. Luckett made a renewed visit to the Central Embryological Collection during April and May. He studied slides from the Hubrecht and Hill collections relating to the early development of the placenta and foetal membranes in South-American monkeys and the gibbon. The results will be incorporated into a chapter currently in preparation for "Advances in Primatology".

2. Dr. H. Grüneberg visited the Central Embryological Collection for several days in August to study sections of the nasal region of various mammalian embryos.

3. Dr. Madeleine Friant worked in the Central Embryological Collection during the first half of September. She studied the structure of cartilage in slides of embryos of various mammals.

P. D. Nieuwkoop
J. Faber

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CENTRAALBUREAU VOOR SCHIMMELCULTURES BAARN AND DELFT

PROGRESS REPORT 1972

The Centraalbureau voor Schimmelcultures was founded in 1904 by the "Association Internationale des Botanistes". Dr. Johanna Westerdijk at Amsterdam was appointed as the first director in 1907. After the dissolution of the AIB, the Bureau was supported by various Netherlands scientific institutions and associations, esp. by the Royal Netherlands Academy of Arts and Sciences. In 1920 the institute moved to Baarn; the yeast collection has been kept since 1922 at the Laboratory of Microbiology, Technical University, Delft.

After Prof. Westerdijk retired in 1959, she was succeeded as director by Miss A. L. van Beverwijk (1959–1963). In 1964 the CBS moved into a new building in Baarn (Oosterstraat 1). Since 1968, the CBS is an institute of the Royal Netherlands Academy of Arts and Sciences.

The Centraalbureau voor Schimmelcultures maintains a collection of living fungi, yeasts and actinomycetes. In 1971 the total number of strains maintained was 18.000, including 3.500 yeasts. By supplying cultures, identifications and advice to workers in diverse fields of scientific and applied mycology, a service is rendered to all those interested in these micro-organisms. Scientific research is carried out mainly in taxonomy and systematics of fungi. In the division of human and animal mycology, problems pertaining to this field are studied. Investigations on the chemistry of fungal metabolites are carried out in the biochemical department.

Facilities are available to students and guest workers who want to study a mycological subject. Each year courses are given on general and on human and animal mycology.

Scientific Staff (as from December 1st 1972).

Baarn, Oosterstraat 1.
Dr. J. A. von Arx, director (general mycology, Ascomycetes, Melanconiales)
Dr. G. A. de Vries (human and animal mycology, Actinomycetes)
Miss drs. A. C. Stolk (Aspergillus, Penicillium and related genera)
Miss drs. M. A. A. Schipper (Mucorales)
Mrs. drs. E. J. Hermanides-Nijhof (Fusarium, Aureobasidium)
Mrs. drs. A. J. van der Plaats-Niterink (Oomycetes)
Drs. H. A. van der Aa (Sphaeropsidales)
Dr. G. W. van Eijk (biochemistry)
Dr. W. Gams (Verticillium, Acremonium and related genera, Mortierella)
Drs. R. A. Samson (Paecilomyces, Penicillium and related genera)
Drs. G. S. de Hoog (Dematiaceae)
Drs. J. A. J. M. Stalpers (Basidiomycetes)
Mrs. drs. G. de Bruin-Brink (documentation)

Yeast Division, Delft, Julianalaan 67A.
Prof. Dr. T. O. Wikén, Director Laboratory of Microbiology, Technical University
Drs. L. Rodrigues de Miranda (Basidiomycetous yeasts)
Miss drs. M. Th. Smith
D. Yarrow (Saccharomyces and related genera)

1. Division of Fungus Taxonomy

Drs. H. A. van der Aa

Much time was spent on preparing a manuscript on *Phyllosticta*; 47 accepted species are described, 3 of them new. For 12 species the ascigerous state is observed, one of them is not yet described. A few new isolates and several type specimens were studied in this connection. Since the typification of the genus with *Phyllosticta cruenta* (Kunze ex Fr.) Kickx has been clarified, *Phyllostictina* Sydow is considered as a synonym. (DONK, Regnum veget. 34: 11. 1964, Taxon 17: 579. 1968). For the 86 species described in *Phyllostictina* a check list has been prepared. The species excluded from *Phyllosticta* will be listed in a separate paper. *Phyllosticta zeae* Stout from South African corn-leaves was studied both in vivo and in vitro. It appeared to be a *Phoma* species, different from all the species hitherto known in pure culture. The study of this strain and some other interesting *Phoma* isolates is continued in co-operation with Drs. G. H. Boerema, Wageningen. Collection and provisional description of new strains of *Coniothyrium* was also continued. Type strains of *C. juniperi* Moreau & al. and *C. cupressacearum* (Morelet) Morelet were received and compared with the available CBS strains. The first mentioned was identical with *C. fuckelii* Sacc. var. *sporulosum* Gams & Domsch, one of its characters being the formation of a yellow pigment.

Seedlings of *Begonia* species, grown in heavily manured soil, were sent to the CBS for examination of the fungal attack; they were covered with small sclerotia of *Myriococcum praecox* Fr. Isolates of this fungus were compared with CBS strains of *Myriococcum praecox* Fr. and *Papulaspora byssina* Hotson. All proved to be the same species, and *Sclerotium eurotioides* Lib. was found to be another synonym. *Papulaspora thermophila* Fergus of which the type strain CBS 736.70 was examined for comparison, proved to be congeneric with *Myriococcum praecox* Fr., from which it differs mainly by its thermophilic character. *Papulaspora* Preuss (1851) is a later name and, according to WERESUB & LE CLAIR (Can. J. Bot. 49: 2203–2213. 1971), restricted to fungi producing papulaspores, which are defined as thallodic propagules differentiated almost from inception into central and sheathing cells; Fergus' fungus has to be renamed **Myriococcum thermophilum** (Fergus) van der Aa, **comb. nov.** (Basionym: *Papulaspora thermophila* Fergus – Mycologia 63: 426. 1971).

A strain of *Arthrinium phaeospermum* (Corda) M. B. Ellis produced an ascigerous state belonging to the genus *Apiospora*, when exposed to near UV light at 16° C. The ascospores are longer and narrower than in *Apiospora montagnei* Sacc., which has a similar *Arthrinium* conidial state. *Apiospora camptospora* Penz. has comparable ascospores but is connected with the conidial genus *Pteroconium* Sacc. ex Grove (=*Papularia vinosa* (Berk. & Curt.) Mason). All the CBS strains of *Arthrinium phaeospermum* were compared with the new isolate under the same conditions, but none

of them produced the ascigerous state. Clones from ascospores and conidia of the new strain readily produced perithecia. This study has to be supplemented by the examination of the types of some *Apiospora* species.

Lasiodiplodia theobromae (Pat.) Griffon & Maubl., the conidial state of *Botryosphaeria rhodina* (Berk. & Curt.) v. Arx, is a common tropical fungus occurring on various plants. A number of strains sent to the CBS for identification were compared. The variation in conidial shape and size is very wide; one strain recently isolated from *Persea gratissima* has conidia much larger and darker than all the others and appeared to be a different species.

From remains of a dead bird a strain of *Onygena corvina* Alb. & Schw. was isolated, which readily produces ascomatal stromata on common media such as oatmeal, cornmeal and malt agar. The ascocarps have a 5–15 mm long, whitish stalk and a fertile head, 0.3–1.5 mm in diameter, which consists of an outer wall of globose cells and an inner wall of isodiametrical cells, stained brownish towards the centre in which asci develop from croziers; they are thin-walled, 12–15 × 8–12 μm, 8-spored; the ascospores are one-celled, ellipsoidal, brownish, biguttulate, 5–8 × 2–3 μm.

The holotype of *Graphium ulmi* Schwarz, which was supposed to be lost for several decades, was rediscovered in the herbarium A. van Luyk in Utrecht.

Dr. J. A. von Arx

The ontogeny of the conidia in some yeast-like Ascomycetes with septate hyphae was studied. In all species of the genus *Endomyces* and in all but one species of the genus *Dipodascus* the vegetative propagation takes place by the formation of fission cells (arthroconidia). The form genus *Geotrichum* is a representative of this conidial state.

In some species of Ascoideaceae the vegetative propagation takes place by an often bipolar budding with a wide or narrow base, followed by the formation of a cross wall. The conidia are apiculate or truncate at the base or have wide scars at both ends when borne in chains. In *Ambrosiozyma monospora* the conidia are formed in acropetal chains arising simultaneously on often swollen conidiogenous cells (Fig. 1a). In *Ambrosiozyma cicatricosa* the conidiogenous cells are phialide-like, flask-shaped and they elongate during the formation of the conidia (Fig. 1b). Young conidiogenous cells often show annellations, but later they elongate sympodially. In *Botryoascus synnaedendrus* branched conidiophores arise and conidia with a truncate base are formed in sympodulae on indistinct scars (Fig. 1c). This conidial state closely resembles the genus *Raffaelea*, comprising a number of hyphomycetes isolated from bark beetles. The conidial state of *Ambrosiozyma monospora* agrees in many respects with that of *Amorphotheca resinae* Parbery. This conidial state is known as *Cladosporium resinae* (Lindau) de Vries; phylogenetically, however, it is not related

to other species of the genus *Cladosporium*. The conidia are formed in acropetal, often branched chains, and are separated from each other by small denticles or not prominent scars (Fig. 1d). This state requires its own genus name:

Hormoconis v. Arx & de Vries, **nov. gen.**

Coloniae effusae, velutinae, avellaneae vel olivaceae, rapide crescentes; conidiophora ex hyphis repentibus oriunda, erecta, elongata, septata, fusca, in parte superiore ramosa; conidia in catenis ramosis oriunda, ellipsoidea, limoniformia vel cylindracea, utrinque attenuata, saepe denticulata, continua vel uniseptata, straminea vel subhyalina.

Species typica: *Hormodendron resinae* Lindau (Rabenh. Kryptog. Fl. **8**: 699. 1907) = **Hormoconis resinae** (Lindau) v. Arx & de Vries **comb. nov.**

In typical *Cladosporium* species, the catenulate, pigmented conidia are separated from each other by prominent, often thickened or darkened scars. The conidiophores in general are dark, short, basally branched and not elongated as in *Hormoconis resinae*. For *Cladosporium*-like fungi parasitic on leaves, special generic names often are used, such as *Biharia* Thirum. & Mishra, *Fulvia* Ciferri, *Fusicladiopsis* Karakulin & Vasil.= *Karakulinia* Golovina, *Mycovellosiella* Rangel, *Phaeoramularia* Muntañola and *Stenella* Syd. It is not possible to distinguish all these genera on morphological characters and their species had better be classified in *Cladosporium*.

The study of Ascomycetes of the family Gymnoascaceae is continued, partly in co-operation with Dr. G. F. Orr (Dugway, Utah, USA). Many non-ostiolate Pyrenomycetes were compared with ostiolate relatives. It is shown that the presence or absence of an ostiolum, especially in soil-borne and coprophilous groups, often can not be used for the delimitation of genera.

Dr. K. W. Gams

The main project was again the taxonomic study of phialide-forming hyphomycetes with one-celled slimy conidia. In this connection the work on *Gliocladium* and *Verticillium* is steadily being continued. Some interesting strains were obtained from Dr. J. Grinbergs, who isolated them from decaying wood and from soil in Chile. The study of *Coniochaeta* species with *Phialophora* conidial states was continued and yielded some interesting cultures.

From decaying wood near Baarn and in the Teutoburger Wald, W. Germany, several *Chloridium* strains were isolated and studied thoroughly in various stages of cultivation. They could be distributed over at least 8 distinct species. In order to find the correct names, herbarium specimens are being examined. Connected perfect states were found of *Chaetosphaeria myriocarpa* (Fr.) Booth and another still unidentified species of *Chaetosphaeria*. The delimitation of the genus *Chloridium* from *Phialophora*

provides some difficulties. There is a range of *Phialophora*-like cultures with wedge-shaped conidia formed in chains, amongst which the conidial state of *Lasiosphaeria hirsuta* (Fr.) Ces. & de Not. Since in the closely related *L. ovina* (Fr.) Ces. & de Not. similar conidia were found to be produced in slimy heads, this character can not be used to delimit genera, and the species mentioned will be described in *Phialophora*. They are delimited from *Chloridium* by discrete (not integrated) and rather thin-walled phialides.

Mr. J. Mouchacca, Paris, who spent two months at the institute, left a number of *Cladorrhinum* strains isolated from Egyptian desert soil which will allow the distinction of a few more species. One of them was identical with *Bahupaathra samala* Subram. & Lodha; this species is distinguished from *Cladorrhinum foecundissimum* Sacc. & March. by lateral ramification of the conidiophores.

Some species are known to bear phialide-like conidiogenous cells which form only solitary conidia. They were subjected to a detailed study and compared with similar fungi with many-spored phialides. In some cases species with solitary conidia are considered to be closely related to others with multiple conidium formation and the conidiogenous structures are regarded as homologous.

In collaboration with Dr. E. G. Kuhlman, Research Triangle Park, North Carolina, and Chiu-Yuan Chien, Taipei, studies on zygospore formation in *Mortierella* species were continued. Zygospores were found in strains that were preliminarily identified as *M. verticillata* Linnemann. A comparison with all available strains in the CBS collection showed that this species is synonymous with *M. marburgensis* Linnemann; this species is interfertile with *M. humilis* Linnemann. Nevertheless the two species are distinct: in *M. marburgensis* the sporangia contain mostly several spores with a wrinkled surface, while in *M. humilis* sporangioles are always one-spored and have a spinulose surface. These differences could be clearly demonstrated with scanning electron micrographs. In a new species to be described as *M. indohii* Chien invested zygospores were obtained after mating with compatible strains. This fungus produces only stylospores, very similar to those of *M. polycephala* Coemans, and is therefore placed in the section *Polycephala*.

The book "Pilze aus Agrarböden" by K. H. Domsch and W. Gams (1970) was translated into English by P. S. Hudson for publication by Longman, London. Correction of the translation consumed much time.

Prof. Dr. J. Grinbergs
(Guest worker from the Microbiology Department, University of Valdivia, Chile).

A mycological analysis was carried out of rotten wood ("palo podrido" and white rot) of *Nothofagus dombeyi*, *N. obliqua*, *Extoxicum punctatum* and *Eucryphia cordifolia*, collected in rain forests near Valdivia and on

the island of Mocha (Chile). A number of Basidiomycetes were isolated (e.g. *Armillaria mellea*, *Phlebia livida* sensu Burt, *Collybia velutipes* and *Sistotrema brinkmannii*), some Ascomycetes (e.g. *Coniochaeta velutina* and *Chaetomium globosum*), a large number of imperfect fungi, belonging to genera such as *Scytalidium*, *Trichoderma*, *Cylindrocladium*, *Cylindrocarpon*, *Acremonium*, *Gliocladium*, *Paecilomyces* and *Penicillium*, some Mucorales and many, mostly conidial yeasts (*Candida mesenterica*, *C. silvae* and other related, not yet identified species).

In a second project fungi were isolated from forest and agricultural soils from Southern Chile. Besides a large number of common, cosmopolitan fungi, also some interesting strains were encountered which could not be identified with described species. A number of these strains will be the object of future studies by CBS staff members.

Mrs. Drs. E. J. Hermanides-Nijhof

A revision of the genus *Aureobasidium*, based on morphological characters was started. The type species *Aureobasidium pullulans* (de Bary) Arnaud is quite well characterized and has been described from a wide variety of habitats, but it seems likely that several different taxa should be distinguished.

In culture, some Ascomycetes, e.g. species belonging to the genera *Dothiora*, *Pringsheimia*, *Guignardia* and *Potebniamyces* seldom produce ascomata; they usually only form an imperfect state which looks very much like *Aureobasidium pullulans*. It was attempted to distinguish these groups from the common type of *Aureobasidium pullulans*, of which no perfect state is yet known. Much time was spent on examining some thirty strains belonging to species of *Dothiora* and *Pringsheimia*, sent by Dr. L. Froidevaux, Zürich. Further studies were carried out with a large number of strains: those maintained in the CBS-collection, cultures sent for identification and many newly isolated specimens. All strains were studied under different circumstances. In this way a combination of characters was found that can lead to a preliminary grouping. These characters are:

1. Type of conidium-production:
   a. directly on the hyphae
   b. on small denticles
   c. on small side branches
   d. as endoconidia within hyphal cells.
2. Ability of the conidia to form secondary conidia.
3. Size of the conidia: this is very variable, but the average values may be larger in one strain than in another.
4. Size and shape of dark chlamydospores and presence or absence of detached, dark, thick-walled cells.

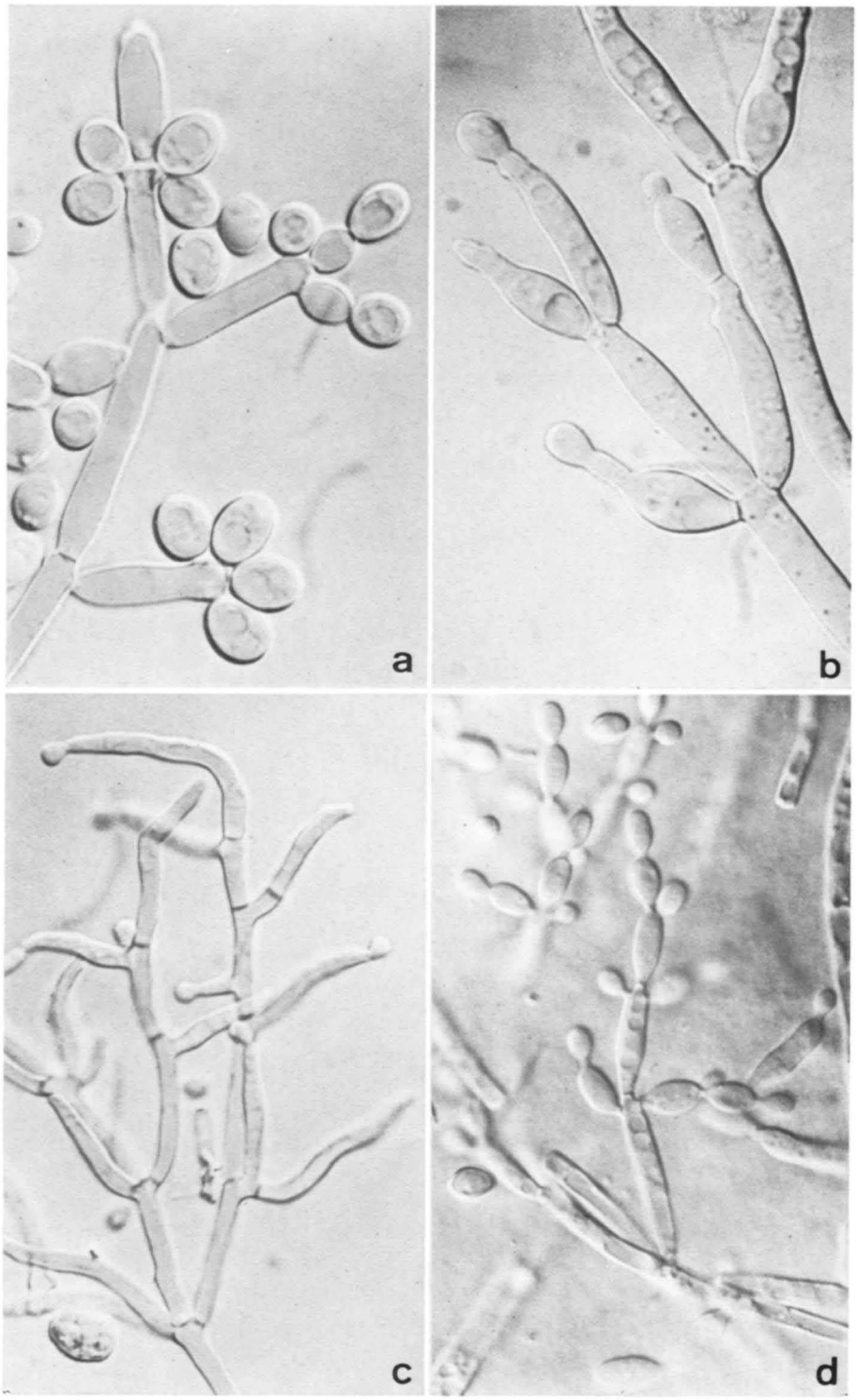


Fig. 1. a. *Ambrosiozyma monospora*, b. *Ambrosiozyma cicatricosa*, c. *Botryoascus synnaedendrus*, d. *Amorphotheca resinae*, conidiogenous cells and conidia; 1200 ×.

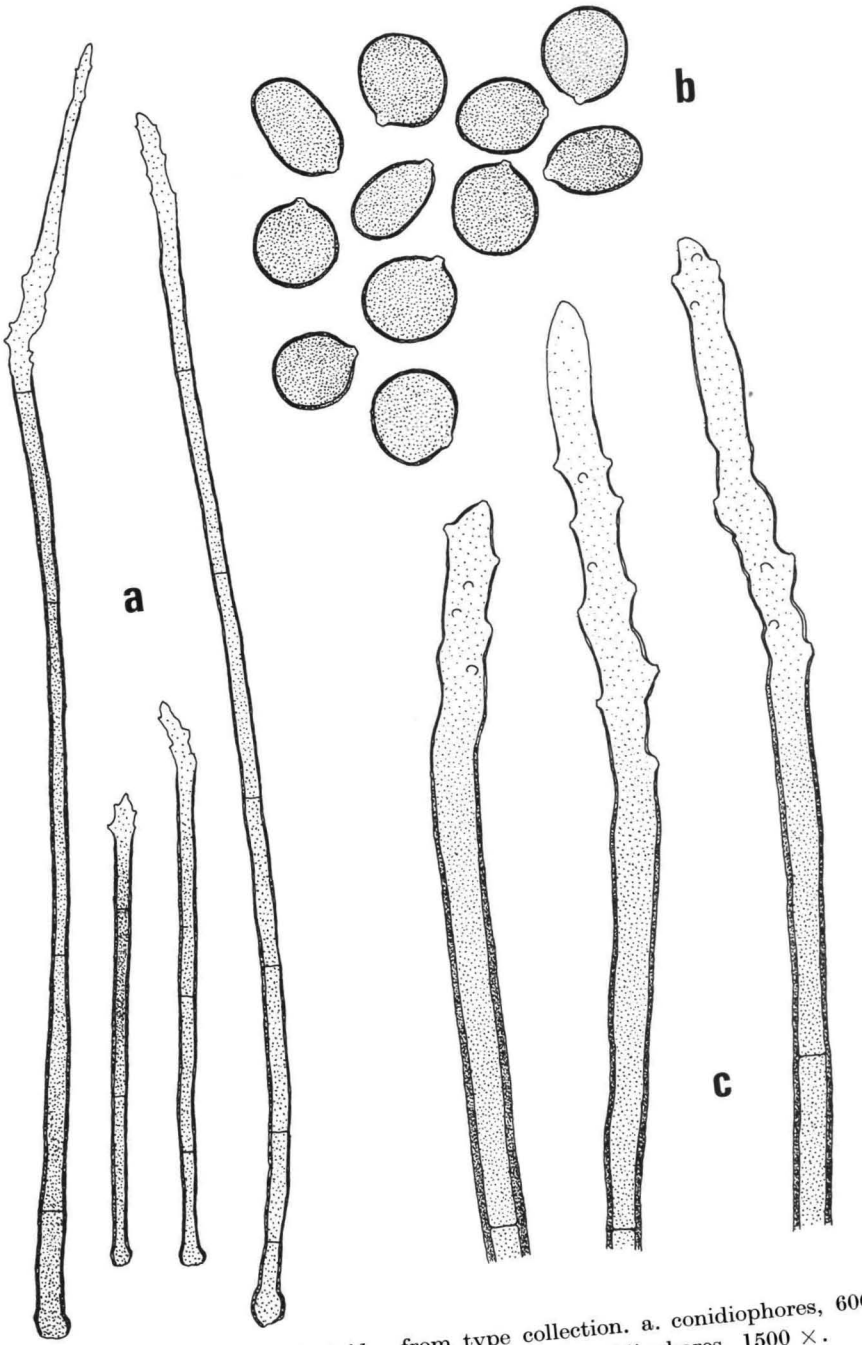


Fig. 2. *Rhinotrichum psilonioides*, from type collection. a. conidiophores, 600 ×; b. conidia, 1500 ×; c. apical structures of conidiophores, 1500 ×.

5. Colour of the culture: although this group of fungi is generally called the "black yeasts" the colour of the different strains can vary from almost white or pink (often with dark spots) via brown, dark green to completely black.
6. Structure of vegetative hyphae.

As yet no clear differences have been found between the species of *Dothiora* and *Pringsheimia*; as a group, however, they seem to be distinguishable from *Aureobasidium pullulans*.

The study of the species belonging to the genus *Fusarium* was continued.

Drs. G. S. de Hoog

Much time was devoted to the preparation of the manuscript on *Beauveria* and similar genera, which was sent to the printer in June. It was published on August 9 as No. 1 in the series "Studies in Mycology".

In *Beauveria* and allied genera good criteria for distinguishing the taxa had been found in the shape of the conidiogenous rachis; similar features were subsequently studied in *Calcarisporium* and *Sporothrix*. In both genera the conidia are formed by sympodial growth of the conidiogenous cell, mostly resulting in more or less globose heads of conidium-bearing denticles. In *Calcarisporium* conidium formation is usually restricted to the apex of the conidiogenous cell; in *Sporothrix* irregular elongation may occur after formation of one or more conidia. Hence, the conidia in *Sporothrix* may arise solitarily or in groups from any place on the conidiogenous cell. However, the line of demarcation between the genera is rather vague; other criteria for distinction, such as yeast stages, deserve more attention.

In some species of *Sporothrix*, besides sympodially formed conidia, blastoconidia are formed at random on the hyphae; mostly they are somewhat thick-walled, brownish, and of rather constant shape and size in every species. On these conidia, which are called chlamydospores by most authors, several species can be distinguished. A number of strains of *Ceratocystis* maintained in the CBS collection have a *Sporothrix* conidial state in pure culture. Some of them are very difficult to distinguish from each other. Attempts are being made to use the variability of the conidia as a taxonomic criterium in some species.

Collections were made of members of the Xylariaceae, especially species of *Hypoxylon*, which have *Nodulisporium* or *Geniculosporium* conidial states. Attempts are being made to correlate perfect and imperfect states, which often are described separately in the literature, by preparing pure cultures of ascigerous states. In this way mostly only the conidial state or a sterile culture is obtained. In several cases sterile stromata are formed in the petri dish; methods have been worked out to induce production of ascospores. Identification of the fungus by means of the conidial state or a sterile culture only is also being attempted. Media for optimal

development were determined. The cultures are preserved in a refrigerator for later analysis. Collections of the fungi on the natural substrate are preserved in the herbarium for reference.

Herbarium materials of some older genera were studied for a revision of more fungi with sympodial conidia similar to *Rhinocladiella* Nannf. During this research particular attention was payed to Preuss' herbarium (B). The type collection of *Rhinotrichum psilonioides* Preuss (Linnaea 24: 121. 1851) was examined. It consists of erect conidiophores, 140–360 μm high and 3–4 μm wide, slightly inflated at the base, apically with an irregularly geniculate, sympodial conidiiferous part. The conidia are brown, smooth-walled, ellipsoidal to subglobose, 8.4–10.3 × 5.2–8.2 μm, often somewhat flattened. The species resembles *Virgariella* Hughes, and should be referred to as **Virgariella psilonioides** (Preuss) de Hoog, **comb. nov.** (Fig. 2).

Jointly with Mr. P. J. Muller (Lisse) a manuscript on the new species *Embellisia hyacinthi* was prepared, which appeared to be connected with a bulbskin disease of hyacinths and some other Liliaceae. Its moderate pathogenicity was confirmed by some German investigators, who isolated the species as well. Meanwhile new strains of *Embellisia* were examined, which do not fit the species already described in this genus.

Dr. Gertrud Franz (Bonn) submitted a culture of a species of *Cordana*, isolated from a Nepalese soil sample (at 1300 m altitude). The fungus appeared to be distinct from all species described in *Cordana* which were available for study. A manuscript was prepared to describe it. During the type studies in this genus, *Cordana bambusae* was examined. The species has percurrent conidiophores, forming apically and sometimes laterally very dark, ellipsoidal conidia. It is similar to *Endophragmiopsis*, described with *E. pirozynskii* as the only species; a new combination in this genus will be proposed.

Dr. S. H. Iqbal
(Guest worker from the Botany Department, Lahore, W-Pakistan).

A few unidentified strains of *Tricellula* were examined with a view to a revision of this genus. A strain of *Volucrispora graminea* Ingold & al. was identified. This species has to be compared with *Ingoldia biappendiculata* and others, before the generic position can be elucidated. The type specimen of *Dactylina* spec. Arnaud was examined and compared with several recent collections of similar fungi. Because of priority of the lichen genus *Dactylina* Nylander, the new name *Dactylinomyces* will be proposed. Several aquatic hyphomycetes were isolated, i.a. *Dendrospora juncicola* Iqbal and *Centrospora* spec. (undescribed).

About 30 collections of ascomycetes growing on submerged branches and herbaceous material were made. Two inoperculate Discomycetes (*Beloniopsis* spec. and *Trichobelonium* spec.) and a *Griphosphaeria* which

appeared to be new species were studied and isolated. Single-spore isolates of the latter formed immature perithecia on 2% malt agar.

Cultures from the CBS and herbarium specimens of *Mollisia* species from the Rijksherbarium Leiden were studied in connection with a monographic work on the Mollisiaceae.

Mrs. Drs. A. J. van der Plaats-Niterink

Much attention was paid to a group of *Pythium* species which in single culture produce filamentous non-swollen or slightly swollen sporangia, but fail to form sex-organs. These strains often readily produce zoospores, especially at temperatures below 20° C. Within the group 4 types of sporangia can be distinguished:

1) non-swollen sporangia, not differing from the vegetative hyphae in appearance.
2) slightly swollen sporangia.
3) slightly swollen sporangia forming slender bunches.
4) slightly swollen sporangia forming rectangularly branched bunches.

Other differences are to be found in the culture pattern, which may be diffuse, radiate or rosette-shaped and in the daily growth rate, varying from 4 to 25 mm at 25° C.

Mating experiments on different media were made with a number of strains with the following characteristics: Sporangia of type 1, a daily growth rate of 7 mm and a rosette culture pattern. Only on a medium of potato-carrot agar to which was added 0.01 ppm cholesterol was there a sexual response. Among 15 strains of this fungus only two strains turned out to be antheridial. In dual cultures the oogonia appeared on short lateral branches of feather-like hyphae of the oogonial strain near the line of contact with the antheridial strain. Many branched antheridial stalks entwine the oogonium in which a single thick-walled oospore is formed. The species has been described as *Pythium flevoense* sp. n. Some mating experiments were started with a number of isolates which showed a daily growth rate of 3–4 mm and a very fine-obtuse rosette culture pattern. Other crossings were started with a great number of strains which in single culture only produced spherical proliferating sporangia. Several media were tried which had yielded results in other mating-experiments: potato-carrot-agar, cornmeal-agar, a mixture of both, hempseed-agar, grass-agar and potato-carrot-cholesterol-agar. Also potato-carrot-agar with varying amounts of a stigmasterol standard emulsion was tried. This standard emulsion was prepared by dissolving 20 mg stigmasterol in a few ml acetone and after adding 50 ml sterilized distilled water, the acetone was evaporated by means of a rotary evaporator. Though on this medium many oogonia were produced by *Pythium flevoense*, no sexual response was got in the other series of experiments.

Concerning the investigation of the occurrence of *Pythium* in the Netherlands, a final series of soil samples was taken from Zuidelijk Flevoland. The number of *Pythium* species in this part of the Netherlands increased in the course of time from 0 in 1967 (1 series of samples), 5 in 1968 (3 series of samples) to 18 in 1970 (3 series of samples). In 1972 12 species were isolated from 1 series of soil samples. In all 23 species of *Pythium* have been isolated from soil from Zuidelijk Flevoland since 1967. A paper about the occurrence of species of *Pythium* in different parts of the Netherlands is being prepared.

Miss Drs. A. C. Stolk

In co-operation with Drs. R. A. Samson the study of the genus *Talaromyces* was completed and a paper prepared. It was published in "Studies in Mycology" as No. 2 on 1st November 1972.

Since the two crozier-producing species originally placed in *Talaromyces* by Benjamin have been transferred to *Hamigera*, *Talaromyces* is now restricted to species with asci borne in chains. However, it is still in some ways a heterogeneous genus. The ascomatal coverings, which consist of networks of hyphae, differ markedly in density in the different species. Ascospores show various patterns. In addition the imperfect states of species of *Talaromyces* vary widely. In *T. byssochlamydoides* and *T. leycettanus* they belong in *Paecilomyces*, those of the other species are to be classified in *Penicillium*, though they belong to quite different series of this genus.

Much attention was paid to the study of the ascomatal initials. In 3 species the initials consist of a pair of gametangia. In the other species antheridia are completely lacking and the initials may consist of subglobose, chlamydospore-like cells; coiled hyphae; swollen, septate hyphae producing coiled branches; or wide, branched, gnarled hyphae. A division of the genus into 4 sections, primarily based on the structure of the conidial state seemed desirable. A correlation proved to exist between the species classified in these sections and their temperature relationships. 16 Species and 2 varieties have been described, illustrated and keyed out. Two new species: *T. byssochlamydoides* and *T. udagawae* and two new varieties: *T. flavus* var. *macrosporus* and *T. helicus* var. *major* were added to the genus. Two species of *Gymnoascus*: *G. flavus* Klöcker and *G. luteus* Sacc. and three species of *Arachniotus*: *A. trachyspermus* Shear, *A. intermedius* Apinis and *A. purpureus* Müller & Pacha-Aue were transferred to *Talaromyces*.

A new series of *Penicillium*, the *P. cylindrosporum* series based on the imperfect species *P. cylindrosporum*, is proposed. It is characterized by biverticillate-asymmetrical, somewhat appressed penicilli with all elements typically roughened, by phialides consisting of a cylindrical basal portion, tapering abruptly to a short but distinct tip and by predominantly cylindrical conidia, colouring the colonies brown or creamish. The new series

includes 4 species: the imperfect species *P. cylindrosporum* and *P. argillaceum* and the imperfect states of *T. bacillisporus* and *T. emersonii*. The species belonging to this series are either thermotolerant or thermophilic.

A manuscript was prepared describing a new species of *Penicillium* isolated from arable soil in Alaska, *P. donkii*. It is characterized by monoverticillate penicilli and soft, brown, sclerotium-like bodies, completely obscuring the brown inner bodies and giving these structures a conspicuous white appearance. The type strain as well as a few additional strains of *P. purpurogenum* var. *rubri-sclerotium* were compared with two strains, regarded by Raper & Thom as representative of *P. funiculosum*. The conidial structures, the rates of growth and the colour of the reverses of the strains examined are similar. As there is no other reason than the production of sclerotia for separating *P. purpurogenum* var. *rubri-sclerotium* and *P. funiculosum*, the mentioned variety of *P. purpurogenum* is considered as a synonym of *P. funiculosum*. The study of the genus *Eupenicillium* was resumed with the examination of a strain received from Mr. R. A. Hill (Rothamsted Experimental Station), which approximates *E. meridianum*, but differs from it in producing slightly smaller ascospores, while the convex surfaces of the ascospores are smooth instead of rough. Hill's strain is being compared with the type strain of *E. meridianum* and a few additional strains, received from Dr. J. P. van der Walt. Moreover the relationships between *E. meridianum* and the sclerotium-producing species of *Penicillium*, *P. indicum*, are being studied.

Drs. R. A. Samson

In co-operation with Miss Drs. A. C. Stolk descriptions and figures for the manuscript on the genus *Talaromyces* were prepared. Examination of the type material of *Arachniotus trachyspermus* (in herb. BPI) proved the identity of this species with *Talaromyces spiculisporus*. In one of the specimens the *Penicillium* conidial state was found. Since the observations with the light microscope did not give enough detailed information about the ornamentations of the ascospores in several species, an additional study was carried out by means of scanning electron microscopy. The *Paecilomyces* conidial states of *Talaromyces leycettanus* and *T. byssochlamydoides* were studied in detail. The imperfect states of both species belong to the *Paecilomyces variotii*-group. In this group the conidial states of the genus *Byssochlamys* and of *Thermoascus crustaceus* are also accommodated.

Many strains of *Paecilomyces elegans*, isolated from different substrates were examined. It was found that the cultural growth of strains isolated from soil is different from those isolated from wood. *Paecilomyces elegans* shows much resemblance to species of the genus *Gliocladium*. Because of the divergent, verticillate conidiophores and catenate conidia, it can be regarded as an intermediate between *Gliocladium* and *Paecilomyces*. The accommodation of *Paecilomyces elegans* in a special section in *Paecilomyces* or in a separate genus is considered.

From Dr. A. von Klopotek (Giessen, Germany) two strains of a new species of the genus *Scopulariopsis*, isolated from self-heated compost were received. A third strain of the same fungus was isolated by Prof. K. H. Domsch in Braunschweig. It belongs to the *Scopulariopsis sphaerospora*-series and is described as *S. murina*. *Paecilomyces fuscatus* Inagaki, which was isolated from food and described as possessing phialides, appeared to produce conidiogenous cells with an annellated zone. The species is therefore transferred to *Scopulariopsis* and the new name *S. gracilis* is proposed.

During the visit of Mr. J. Mouchacca to the CBS, a number of fungi was examined, which were isolated from desert soil in Egypt. Amongst them two interesting species of *Aspergillus* were encountered. One represents a new species belonging to the *Aspergillus nidulans* group; it is related to *A. subsessilis*, but differs from this species by its larger, smooth conidia and the smaller, irregular Hülle cells. Moreover, it has a higher optimal temperature for growth (35° C) and is very osmophilic. The other species is very similar to *Emericella fruticulosa*, differing only in some cultural aspects. *E. fruticulosa* is only known by its type culture, which was also isolated from desert soil. A manuscript with descriptions of both species from Egyptian desert is in preparation.

Much time was spent on the identification of *Penicillium*-like fungi isolated by Prof. J. Grinbergs (Valdivia, Chile) from Chilean soil and by Dr. H. Fiedler (Krefeld, Germany) from meat and sausages. Herbarium material and pure cultures of entomogenous fungi were sent by Dr. H. C. Evans (Tafo, Ghana). These fungi were collected in the rain-forests in Ghana. Most of them could be tentatively identified as species of the genera *Akanthomyces*, *Insecticola*, *Hirsutella*, *Gibellula* and others, but several specimens probably represent undescribed taxa. *Gibellula formicarum* Mains appeared very commonly on different insects in Ghana. The species was only known by its type specimens on an ant collected in Liberia. The attempts at cultivation of other species of the genus *Gibellula* failed, only *G. formicarum* could be isolated in pure culture. Some species, previously described as *Isaria* were also collected by Dr. H. C. Evans and cultivated on agar media for the first time. Observations of the fungi on their natural substrate (insects) and in pure culture revealed, that these species are closely related to *Paecilomyces farinosus*.

Drs. J. A. J. M. Stalpers

The study on the cultural characters of wood-attacking Aphyllophorales was continued. Much material was collected in the surroundings of Soest and Baarn and in the Teutoburger Wald (W-Germany). More than 60% of the species could be cultivated. Also many of the CBS-strains have been examined. The punch card system for mycelial characters was augmented. Because there is no recent monograph and the descriptions

are scattered, a key comprising the European species of most subfamilies of the Corticiaceae has been compiled from the literature.

A taxonomic study of the genus *Oedocephalum* Preuss was started. The form genus *Oedocephalum* is characterized by rather wide conidiophores with an apical swelling on which hyaline blastoconidia are produced simultaneously on denticles. Its perfect states partly belong to Ascomycetes (Pezizaceae), partly to Basidiomycetes (Corticiaceae and Polyporaceae). 42 *Oedocephalum* species have been described (exclusive of unnamed conidial states of Ascomycetes and Basidiomycetes), 12 have to be excluded and belong to such different groups as Uredinales, Mucorales and *Aspergillus*, and most of the remainder have to be placed in synonymy.

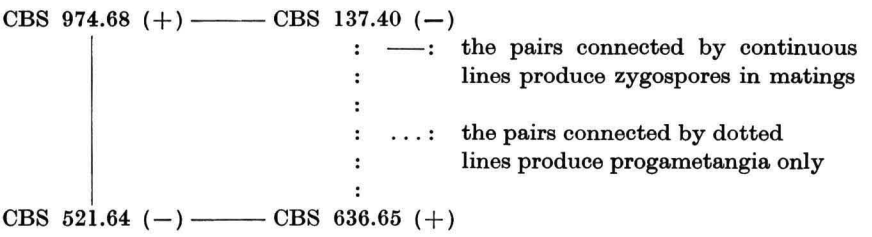
O. glomerulosum (Bull.) Sacc. is usually designated as the type species, because *O. elegans* Preuss (the first of the three species originally described by Preuss) was regarded as a synonym of this species by Harz, and subsequently by Saccardo and Clements & Shear. A study of the type specimen proved *O. elegans* to be a different species, afterwards described by Jaap as *O. griseobrunneum*.

The type specimen of *O. sulphureum*, growing on rope, was identified as a species of *Oidiodendron*, closely related to *O. flavum* Szilvinyi emend. Barron. The fungus is sulphur-yellow on the natural substrate, the conidiophores are rather short, with a pigmented, sometimes roughened basal part, and hyaline, often curved, dense branches in the upper part. The branches divide into yellowish, smooth or roughened arthroconidia, ovoid-ellipsoid, often connected by thin-walled, rather wide sterile segments. The species has to be renamed **Oidiodendron sulphureum** (Cooke & Massee) Stalpers **comb. nov.** (basionym: *Oedocephalum sulphureum* Cooke & Massee – Grevillea **17**: 3. 1888).

Miss Drs. M. A. A. Schipper

The study of the genus *Mucor*, aimed at grouping the species in a natural system, is still in progress. The morphology of a large number of cultures belonging to the species *M. saturninus*, *M. mucedo*, *M. piriformis*, *M. wosnessenskii* and *M. plasmaticus* was studied on various media and at various temperatures. Intra- and interspecific matings were made with these strains. Though the successful mating of compatible *M. saturninus* strains was reported in 1969 (CBS Progress Report), a description of the zygosporic stage was not given. The following description is based on a mating of CBS 974.68 (+) and CBS 137.40 (−) on beerwort agar at 15° C. The zygospores, formed in the aerial mycelium near but not on the medium, are globose to slightly compressed between suspensors, up to 180 × 170 μm, with protuberances up to 10–11 μm in length, black; the suspensors, equal or unequal, are up to 55 μm in diam., with or without reddish to yellowish contents; the zygophores, not quite equal, are up to 17 μm in diam. and have encrusted walls. 16 Strains, similar in general morphology, were mated with the tester pair and with each other. Not

all strains formed zygospores when paired with a tester; in matings with other strains of the species, the conjugation was complete.



Similar mating-results were obtained in other species of the genus *Mucor*, e.g. in *M. hiemalis* (Schipper: A study on variability in *Mucor hiemalis*, in preparation).

The zygosporic stage in *M. mucedo* is well known. It is the most studied conjugation in the genus *Mucor*. And yet sexually active strains of *M. mucedo* are rare. The strains used by BURGEFF (1924), originating from K. Saito, Japan, CBS 109.16 and CBS 144.24, are still the mating partners for all research and demonstration work. Some recent isolates of *M. mucedo* were found to be able to produce at least a small number of zygospores on mating with the above-mentioned strains. The strains CBS 109.16 and CBS 144.24, though successful mating partners, are in a rather poor condition in so far as asexual reproduction is concerned: few sporangiophores and sporangia are produced. The recent isolates form a well developed layer of short, repeatedly branched sporangiophores, rather sparsely mixed with tall sporangiophores that are either simple or produce short, repeatedly branched sporangiophores near the base, while large branches infrequently occur equally inserted near the basal part of the sporangiophore.

The zygospores of *Parasitella simplex*, a facultative parasite on other Mucorineae, have been described by BURGEFF (1924). Burgeff did not find zygospores in matings of pure cultures, but when parasitizing other Mucorineae the mated strains did produce zygospores. SATINA & BLAKESLEE (1926) obtained zygospores in matings of pure cultures, but were unable to determine the mating-reaction types of the strains, since in matings with the known (+) and (−) strains of other Mucorineae no recognizable sexual responses were observed.

In matings of monosporangial cultures of CBS 412.66, *Parasitella simplex* and CBS 243.35, *M. luteus* (−) reddish brown fruit bodies of the *Parasitella simplex* type were formed, either zygospores or azygospores. Since it is rather difficult to free the fruit bodies of the adjacent hyphal growth, and only one suspensor was seen, there is no proof of conjugation. But, whether due to mating or only an induction, this result shows that most probably the strain CBS 412.66 has a (+) mating reaction.

2. Division of Biochemistry

Dr. G. W. van Eijk

A study of the carotenoid pigments of *Arthrobotrys superba* CBS 662.70 is going on. Twenty-five days old cultures of the fungus grown on oatmeal agar medium in petri-dishes were extracted with methanol. The unresolved carotenoid mixtures were separated on thin-layer chromatograms after preliminary phase separation and saponification. Carotenoids were analyzed spectrophotometrically. The methods used have been described in detail by VAN EIJK (Antonie van Leeuwenhoek 38: 163. 1972). Several carotenoids were isolated. Four of them were identified as the acidic pigment neurosporoxanthin, γ-carotene, β-carotene and torulene. These compounds were also found in *Arthrobotrys oligospora* by VALADON (Phytochemistry 2: 103. 1963). The nature of some minor carotenoids of *A. superba* is under investigation. The presence of ergosterol in the mycelium was proved.

The pigments of a particularly pigmented strain of *Talaromyces stipitatus* (CBS 349.72) were isolated and identified. Cultures of the fungus grown on Czapek-Dox agar were extracted with ethyl acetate. The residue obtained after evaporation was extracted twice with light petroleum b.p. 40–60° C and then recrystallized from chloroform-methanol (2:1). Bright red needles were obtained. The compound was identified as erythroglaucin (1,4,8-trihydroxy-3-methyl-6-methoxyanthraquinone) by physico-chemical methods. The finding was confirmed by direct comparison with an authentic sample obtained from *Eurotium rubrum* CBS 110.31. From the mother liquor two other anthraquinone derivatives were isolated by means of preparative thin-layer chromatography. One compound turned out to be catenarin (1,4,6,8-tetrahydroxy-3-methylanthraquinone) and showed complete agreement in all respects with catenarin isolated from *Drechslera catenaria* CBS 191.29. The other substance could be identified as emodin (1,6,8-trihydroxy-3-methylanthraquinone) by direct comparison with a commercial sample. A manuscript on this investigation has been submitted for publication. *Talaromyces stipitatus* strains are known to produce the tropolones stipitatic acid and stipitatonic acid. No evidence has been obtained for such production by the strain CBS 349.72. Both acids were isolated from the culture fluid of another strain examined (CBS 227.72).

The study of the structure of the red pigments of *Arthrinium phaeospermum* (CBS 142.55, type culture of *Botryoconis sanguinea*) was continued. The structure of an anthraquinone with three α-hydroxy groups was previously assigned to the metabolite (CBS progress report 1971). However, evidence for the presence of a naphthazarin-type nucleus in the molecule of the pigment has now been provided by further experiments. Acetylation of the pigment with acetic anhydride and a trace of conc. H_2SO_4 afforded several acetates. The structure of these is under investigation. It was observed that a particular strain of *Penicillium* yielded

an abundant quantity of crystals in the agar of various media. Chemical and spectroscopic examination of the isolated and purified crystalline compound showed that it was the antibiotic griseofulvin.

A study of the cell-wall components of yeast-like fungi was started. Many difficulties were encountered with the preparation of cell-wall material. Finally, an excellent disruption of the spherical cells was obtained with glass beads 0.45–0.50 mm in a Braun cell homogenizer. Efforts are now directed for collecting a cell-wall fraction without cytoplasmic contamination by repeated sonication, centrifugation and washing.

3. Division of Human and Animal Mycology

Dr. G. A. de Vries

A manuscript on what in June 1971 was regarded as the first case of Lobo's disease in a dolphin (*Sotalia guianensis*) has been revised, as it appeared that MIGAKI, IRVINE & GARNER (J. Am. Vet. Med. Ass. 159, 5: 578–582. 1971) had already discovered this disease in an Atlantic Bottle-Nosed Dolphin (*Tursiops truncatus*) caught in Florida. The discovery of the same disease in *Sotalia guianensis* in Surinam remains important not only because it is the first record of this mycosis in this species and the second in a dolphin but also because the animal was caught in the area of distribution of Lobo's disease in man (Borelli's "reservarea"). Two fungi isolated from the skin specimens, which was heavily contaminated with bacteria, were identified as *Glenospora graphii* Vuill. and *Torulopsis haemulonii* van Uden & Kolipinski.

The latter species, which was identified by D. Yarrow at Delft, represents the third record of this yeast in the world. Neither of the two fungi was regarded as the etiological agent. A manuscript describing the Sotalia disease was sent to the editor of "Aquatic Mammals".

Cultural investigations into a blue-pigment-producing deuteromycete isolated from human hair and skin were continued. Experiments carried out at the Universiteitskliniek voor Huidziekten at Utrecht proved that the three strains of the fungus did not cause any lesion in the skin of guinea pigs.

In co-operation with Miss C. R. Josephus Jitta of the Universiteitskliniek voor Huid- en Geslachtsziekten at Amsterdam a study was made of a small epizootic among horses in the Netherlands caused by *Trichophyton equinum* var. *equinum*. A manuscript in which this epizootic is described was submitted to Sabouraudia.

To expedite the diagnosis of *Cryptococcus neoformans* it was considered to be appropriate to use the discovery made by BARTSCH & STAIB (Naturwissenschaften **52**, 16: 477. 1965) that colonies of *Cryptococcus neoformans* turn brown on media with an extract of the green parts of several Compositae. Therefore such an extract was prepared from *Taraxacum officinale* and *Hypochaeris radicata*. This extract was added in various concentrations

to agar media. The results were in accordance with Bartsch & Staib's findings. Although the colonies varied considerably in the intensity of pigmentation, all were darker than those grown on the control media without extract.

In May, August and September 90 soil samples were collected in Southern and Eastern Flevoland. With a selective technique 2 colonies of the keratinolytic *Arthroderma uncinatum* (st. con. *Trichophyton ajelloi*) were isolated from samples collected in a potato field in Eastern Flevoland. Among the thermophilic and thermotolerant fungi, the pathogen *Aspergillus fumigatus* was the commonest species. *Thermoactinomyces* species were also often isolated. The antimycotic activity of 23 thermotolerant *Streptomyces* strains was investigated using *Trichophyton mentagrophytes* and *T. rubrum* as test organisms. It appeared that three strains belonging to one *Streptomyces* species strongly inhibited the dermatophytes. The activity was, however, only observed when both the *Streptomyces* and the *Trichophyton* were growing on the same agar plate. Cell-free extracts have so far not shown any activity at all.

4. **Division of Yeasts** (Laboratory of Microbiology, Delft)

Head: Prof. Dr. T. O. Wikén

Drs. L. Rodrigues de Miranda

The study of a strain of *Candida tropicalis* CBS 6418 as described in the 1971 report was continued in co-operation with Mrs Dr. S. Sukroongreung.

The behaviour of the nuclei during the various macroscopically and microscopically distinguishable stages was further investigated, namely:

1) The active unstable haplophase. It is characterized by a very small flat type of colony composed of relatively small cells.

2) The diplophase, which can be macroscopically distinguished by the dome-shaped colonies arising out of the flat small active haploid colonies. The cells are considerably larger than those of the haplophase and giant cells are frequently encountered.

3) The inactive haplophase. This is the stable phase; it arises from the diploid colonies either directly after inoculation or after some time. The colony form is usually irregular with very spreading growth. In addition this form can arise directly from active haploid cells. Plating out an active haploid colony always results in three kinds of colonies, active haploid, diploid, and inactive haploid.

The behaviour of the nuclei was studied by means of preparations stained by the Giemsa method. Because the chromosomes of most strains of *Candida tropicalis* can be made visible fairly easily, in contrast to e.g. those of *Candida albicans*, it was possible to distinguish haploid, diploid

and polyploid nuclei. The number of chromosomes in the haploid nucleus of *Candida tropicalis* is probably 6 like in *Lipomyces lipofer* (ROBINOW, J. biophys. biochem. Cytol. 9: 879–892. 1961). The active haploid nucleus can give rise either to an inactive haploid nucleus or by autodiploidization (duplication of the chromosomes in the cell itself) to a diploid nucleus.

A mitotic division and a meiosis could be distinguished. During mitosis the following phases could be recognized:

1) resting nucleus, dark, compact, without regular structure.
2) early prophase. The chromosomes can be made visible (6 per nucleus).
3) late prophase and early metaphase. The chromosomes are duplicated (11 to 12).
4) late metaphase and anaphase. The two sets of chromosomes are moving apart.
5) Telophase. The connections between the two sets break and the two daughter nuclei move to the opposite poles. The cell walls of the two daughter cells are completed.

Meiosis could be shown in cells from diploid colonies of 15 hours to 10 days old. The different distinguishable steps are in sequence:

1) Early prophase with chromosome number $2n = 12$.
2) Late prophase and metaphase. The nuclei expand to large globules in which the number of chromosomes is difficult to count because of overlapping. We have counted approximately 22–24 chromosomes per nucleus.
3) During anaphase the nucleus devides into two parts each containing 2n chromosomes. One part migrates into a bud and the other half remains in the mothercell.
4) During the following steps named interphase and second anaphase the reduction division takes place. Either the two cells of the first anaphase bud again, resulting in 4 new daughtercells, each containing one haploid nucleus or the first bud retains its broad connection with the mothercell and the four nuclei with n chromosomes remain in the two cells with open connection. Later each nucleus moves one by one into a new bud until there is only one nucleus in every cell. We have introduced the term "budding meiosis".

Besides this type of meiosis there is another unexpected way of meiosis. Nuclei with approximately 6n chromosomes were found. During the first anaphase three 2n nuclei are formed which remain in the mothercell. In the second anaphase each 2n nucleus divides into two haploid nuclei which results in a cell with 6 haploid nuclei. At the end these nuclei migrate to buds. A second uncommon phenomenon is the unequal division of triploid nuclei into a haploid and a diploid nucleus.

A similar phenomenon was found in *Syringospora albicans*.

At the time of duplication of the chromosomes the cells of some strains of *Candida tropicalis* and of *Syringospora albicans* enlarge, resulting in thinwalled giant cells. These cells can definitely not be considered as chlamydospores. They contain four or more spores (see under no. 4, description of budding meiosis). The maximum number that is found is 16. They cannot either be considered as metabasidia, since basidiospores on sterigmata like VAN DER WALT (Antonie van Leeuwenhoek **35**: 246–256. 1967; Mycopath. Mycol. appl. **40**: 231–243. 1970) described for *Syringospora albicans*, were not found.

Chlamydospores. Two types of chlamydospores were found in *Candida tropicalis* CBS 6418: free chlamydospores without connections with single cells or mycelium and chlamydospores on mycelium. The number of chromosomes in the single nucleus of the chlamydospores is about $n=6$; no evidence was found that meiosis takes place in the chlamydospores. The maximum number of chromosomes found was $2n=12$, namely just before germination with a mitotic division. Therefore chlamydospores cannot be considered as teliospores, but are true resting spores. Chlamydospores produce sexually inactive and sexually active haploid buds which can be separated by plating out.

There are several arguments against the disposition of *Candida albicans (Syringospora albicans)* in the group of yeasts related to the Hemibasidiomycetes.

1) KREGER-VAN RIJ and VEENHUIS (J. gen. Microbiol. **69**: 87–95. 1971) found the cell wall structure of *Candida albicans* to be related with that of ascomycetous yeasts.

2) The guanine-cytosine content of the DNA is between 35.1 and 36.9 for *C. albicans* and 34.9–35.0 for *C. tropicalis*.

Yeasts with a GC content of their DNA under 50% are considered to be related to the Hemiascomycetidae, above 50% to the Heterobasidiomycetes.

3) The polynucleate giant cells resemble the polynucleate stages of some ascomycetous yeasts like *Lipomyces lipofer* and *Kluyveromyces polysporus*, just before the walls of the ascospores are formed.

A paper, illustrated with diagrams and photographs will be published in 1973 in volume 39 of Antonie van Leeuwenhoek.

Miss Drs. M. Th. Smith

The strain CBS 6367, received from the NRRL labelled *Schwanniomyces occidentalis* NRRL Y–1287, was checked. It differed in some physiological characters from the standard description of *S. occidentalis* (The Yeasts, 1970). L-sorbose, cellobiose, salicin, succinic and citric acids are assimilated and maltose is fermented. The assimilation and fermentation patterns show more resemblance to the standard description of *Schwanniomyces*

alluvius. However, this species should assimilate and ferment melibiose and 6367 does not. In order to see whether this was possibly an intermediate between *S. alluvius* and *S. occidentalis*, all the strains of both species were examined. The physiological characteristics observed in the type strain CBS 4516 and two other strains CBS 4668 and 5655 agree with the standard description of *S. alluvius*. Both strains of *S. occidentalis* (type strain CBS 819 and CBS 1153) assimilate L-sorbose, cellobiose, salicin, succinic and citric acids and ferment maltose. The standard description in The Yeasts, 1970, is apparently incorrect. CBS 6367 can be identified with *S. occidentalis*.

In 1960 Ohara & al. described the yeast strain 0–7 (=CBS 4077=IFO 1092) isolated from grape must as *Candida vinaria* nom. nud. (J. agric. chem. Soc. Japan **24**: 709. 1960). According to them *C. vinaria* resembles *C. rugosa*, especially in its physiological properties, but differs from it by "rare formation of pseudomycelium, poor growth in malt extract, absence of pellicle, smaller and shorter cells". *C. vinaria* was placed in synonymy with *C. zeylanoides* by Buckley and van Uden (The Yeasts, 1970, p. 1078). CBS 4077 was compared with the type strain of *C. rugosa* (CBS 613) and of *C. zeylanoides* (CBS 619). In malt extract short oval cells measuring 4.0–5.0 × 2.0–3.0 μm and narrow elongate cells, measuring 5.0–12 × 1.5–2.0 μm, were observed in CBS 4077. The cells of CBS 613 are more oval to elongate and measure 7.0–13.5 × 2.0–3.5 μm. The cells of CBS 619 are ovoid and measure 7.5–10.5 × 3.0–6.0 μm. In slide cultures on cornmeal agar strain 619 forms a well developed mycelium consisting of branched, robust pseudohyphae with few or no blastospores. Strain 613 forms a primitive pseudomycelium that consists of rarely branched, long chains of elongate cells. CBS 4077 forms only a sparse primitive pseudomycelium consisting of short unbranched chains of narrow, elongate cells.

NAKASE & KOMAGATA (J. gen. appl. Microbiol. **17**: 259. 1971) found the GC content of *C. zeylanoides* (CBS 619) to be 55.9%, that of *C. rugosa* (CBS 613) to be 50.2% and that of *C. vinaria* (CBS 4077) to be 44.1%. In view of the differences in GC content, the cell size and ability to produce pseudomycelium, CBS 4077 can indeed be considered a separate species and the name is validated.

Candida vinaria Ohara, Nonomura & Yunome ex M. Th. Smith, **spec. nov.**

In extracto malti post 3 dies cellulae breves ellipsoidales, 4.0–5.0 × 2.0–3.0 μm et longae, 5.0–12.0 × 1.5–2.0 μm, formantur, singulae vel binae vel in catenis brevibus. Sedimentum et anulus formantur. In agaro zeae maydis post 7 dies pseudomycelium paucum formatur. Fermentatio nulla. Glucosum, galactosum, L-sorbosum, D-xylosum, D-ribosum, aethanolum, glycerolum, D-mannitolum, sorbitolum, acidum succinicum, acidum citricum assimilantur, non vero sucrosum, maltosum, lactosum, cellobiosum, trehalosum, melibiosum, raffinosum, melezitosum, inulinum, amylum, L-arabinosum, D-arabinosum, L-rhamnosum, erythritolum, adonitolum, dulcitolum, α-methyl-D-glucosidum, salicinum, acidum lacticum, inositolum. Kalii nitratum non assimilatur.

Cultura typica e musto uvarum isolata CBS 4077.

The strains CBS 2932 (=NRRL Y-2908-5) and CBS 2933 (=NRRL Y-2908-12) were placed in the collection in 1957 under the genus *Endomycopsis* as the undescribed species *Endomycopsis rettgeri*. As was shown by VAN DER WALT & SCOTT (Mycopath. Mycol. appl. 73: 279. 1971), the generic name *Endomycopsis* is illegitimate and all the species belonging to this genus have to be transferred to other genera. The strains CBS 2932 and 2933 were re-examined. It was known that 2932 and 2933 were mating types. The fermentation and assimilation pattern of these strains is the same as that of the type strain of *Pichia etchellsii* (CBS 2011). In malt extract the cells of 2932 and 2933 measure 5.5–12 × 3.0–5.5 μm, those of the type strain of *P. etchellsii* 5.0–10 × 3.0–5.5 μm. However, *P. etchellsii* forms spherical ascospores (1–4 per ascus) after conjugation with the bud, whereas in *E. rettgeri* they are hat-shaped.

In *Pichia membranaefaciens* some strains are homothallic; conjugation between mother cell and bud has been observed. According to SLOOFF (Antonie van Leeuwenhoek 30: 129. 1964) other strains of this species may be heterothallic. Moreover experiments of N. J. W. KREGER-VAN RIJ (A Taxonomic Study of the Yeast Genera Endomycopsis, Pichia and Debaryomyces, Thesis, Leiden. 1964) revealed that a strain which after conjugation with its bud forms spherical spores, gives hat-shaped spores after mating with one of the mating types. To find out whether such a phenomenon can occur in *P. etchellsii*, attempts were made to cross the four strains of *P. etchellsii* (CBS 2011, 2012, 5519 and 5603) with the strains 2932 and 2933. These experiments have still not yielded any results; neither conjugation nor ascospores were observed.

The fermentation and assimilation patterns of the two strains examined show a great resemblance to the pattern of *Candida sake*. A second series of mating tests of 2932 and 2933 with all our *C. sake* strains was commenced to see whether any are related to *P. etchellsii*.

D. Yarrow

The six strains labelled *Hormoascus platypodis* in the collection were examined. The type strain CBS 4111, a single-spore isolate from it CBS 4380, and two isolates from tunnels of ambrosia beetles in South Africa, CBS 6110 and CBS 6164, are morphologically identical. There were some physiological differences between the strains. According to the standard description given in The Yeasts, 1970, based on the strains 4111, 5560 and 5561, soluble starch is not assimilated and only glucose is fermented. Strains 4111 and 4380 were able to assimilate soluble starch, giving a maximum reading within two weeks of agitated cultures, and 4380 fermented sucrose weakly in the third week of incubation. Strains 6110 and 6164 did not assimilate soluble starch but they fermented sucrose more vigorously than strain 4380, they also fermented maltose weakly towards the end of the third week. Despite these minor physiological differences all four strains are retained in *Hormoascus platypodis* and the

description of the species should be emended to: fermentation of sucrose and maltose weakly positive or negative, assimilation of soluble starch positive or negative. The two other strains, CBS 5560 (=NRRL Y–6106) isolated from the South Santiam river in Oregon, and CBS 5561 (=NRRL Y–4169) isolated from frass of *Tsuga canadensis* in New Hampshire, cannot be distinguished physiologically from *H. platypodis*. Neither strain assimilated soluble starch and both fermented sucrose weakly and latently. However, the cells of these strains were smaller. In malt extract they measured 3.5–6 × 3–6 μm whereas those of the four *H. platypodis* strains measured 4–9 × 4–8 μm. NAKASE & KOMAGATA (J. gen. appl. Microbiol. **17**: 77. 1971) found the GC content of the DNA of *H. platypodis* CBS 4111 to be 36.6–36.8%, whereas they found that of CBS 5560 and CBS 5561 to be 31.5–32.7%. In view of the differences in GC content and cell size CBS 5560 and CBS 5561 can be considered representatives of a second species of *Hormoascus*.

The strain of *Saccharomyces florenzani*, CBS 6339, deposited in the collection by G. Florenzano, was found to have cells, asci and ascospores identical with those of *Saccharomyces rosei* and it probably represents yet another physiological race of this species. In addition, this strain is identical to a spontaneous, melibiose-fermentative mutant isolated from *S. vafer* CBS 6105. (The original spelling florenzanii is regarded as an orthographic error and has been corrected to florenzani).

The strain CBS 5945 (=IFO 0035) listed as *Saccharomyces bisporus* was found to differ from the other strains of this species by assimilating succinic acid but not glycerol; on glucose-peptone-yeast extract agar its cells were globose or nearly so, whereas those of the true *S. bisporus* strains were ovoidal to ellipsoidal. TSUCHIYA et al. (Jap. J. exp. Med. **37**: 285. 1967) described this strain as *Pichia krusei* (nom. nud.), distinguishing it from *S. bisporus* serologically. As it had the same antigenic structure as *Candida krusei* they considered it to be the perfect form of this species. Nakase & Komagata (l.c.) found the GC content of the DNA of the type strain of *S. bisporus* to be 44.1% and that of CBS 5945 to be 38.5%. These authors maintained *P. krusei* as a species and, on the basis of GC content and carbon assimilation pattern, identified the strain CBS 794 with it. Morphological comparison of the two strains confirmed the above authors' opinion of their identity, on glucose-peptone-yeast extract-agar the cells of both strains are globose to subglobose, 4–7 × 3.5–5 μm. However, as CBS 794 is the type strain of *Pichia dispora*, this epithet has priority over *P. krusei*, which in any case is a nomen nudum.

The type strain of *Candida fibrae* Nakase, CBS 6375, was found to be identical with *Pichia burtonii* and formed ascospores when mated with the strain CBS 2352.

Two strains of *Saccharomyces castellii* Capriotti, a species for which a type was not indicated, CBS 4309 and CBS 4310 isolated from soil in

Finland by A. Capriotti, could not be distinguished from *Saccharomyces dairensis* as described in The Yeasts, 1970.

Two new *Torulopsis* species described by KOCKOVÁ-KRATOCHVÍLOVÁ & ONDRUSOVÁ (Biológia, Bratisl. 26: 477–458. 1971) were found to produce pseudohyphae abundantly and therefore should be classified in the genus *Candida*. *Torulopsis schatavii* is physiologically very similar to *Candida cacaoi*, *C. boleticola* and *C.c onglobata*, but differs from all of these by failing to use β-glucosides as sole source of carbon. *Torulopsis kruisii* is superficially similar to *Candida viswanathii*.

Towards the end of the year some pink and red auxothrophic mutants were obtained from several *Saccharomyces* species by treatment with N-methyl-N'-nitro-N-nitrosoguanidine. It is hoped that these will facilitate future hybridization experiments with species other than *S. cerevisiae*.

J. A. VON ARX

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INSTITUTE OF ECOLOGICAL RESEARCH

PROGRESS REPORT 1972

1. History and function of the institute

The institute was founded in 1954 by the Division of Sciences of the Netherlands Academy of Sciences on the initiative of the Committee on Ecology. This proposal was based on the lag in some aspects of ecological research in The Netherlands noted by the committee.

The function of the institute is to perform and to encourage terrestrial ecological research in a broad sense and to co-operate with other organizations engaged in such research.

At the start, several already existing institutes of scientific research were incorporated, viz. the Bird Migration Station, the Bird-Ringing Department of the National Museum of Natural History at Leiden, and the Weevers' Duin Biologica. Field Station of the Foundation for Scientific Dune Research. The population research on the Great Tit, which had been carried out until then by the Phytopathological Service at Wageningen, was also taken over. The distributional research on organisms in the reclaimed IJsselmeer polders was started after the foundation of the institute.

The headquarters of the institute are at Arnhem; the Department of Botanical Ecology has its seat at Oostvoorne. The field work locations are indicated in Fig. 1.

The institute is supervized by a committee appointed by the Division of Sciences of the Academy.

The address of the institute is: Kemperbergerweg 11, Arnhem, Holland.

2. Scientific Staff

J. W. Woldendorp (Director)

Population ecology
J. H. van Balen (Head, Ecology)
J. A. L. Mertens (Physiology)
H. N. Kluyver (Guest worker)

Bird migration
A. C. Perdeck (Head, Bird Migration)
A. J. Cavé (Orientation)

Distributional ecology
J. H. Mook (Head, Zoology)
J. Haeck (Zoology)
R. Hengeveld (Zoology)
J. van der Toorn (Botany)

Botanical ecology Weevers' Duin, Oostvoorne
M. J. Adriani (Head of Department)
Ph. Stoutjesdijk (Micrometeorology), from 1 June 1973 at Arnhem
A. H. J. Freijsen (Experimental Ecology)
C. P. W. M. Blom (Experimental Ecology)
P. A. I. Oremus (Microbiological Ecology)
C. van Dijk (Microbiological Ecology)
D. van der Laan (Synecology)
J. H. Wessels (Soil analysis)

Fig. 1. Geographical position of the Institute of Ecological Research and its field work sites.

1. Headquarters at Arnhem.
2. National Park *De Hoge Veluwe*, main scene of the field work on the Great Tit.
3. Ermelo, main scene of the field work on the Collared Dove.
4. Vlieland, additional field work site of the Departments of Population Ecology and Bird Migration.
5. Westeinder Plassen, main scene of the field work on the Coot.
6. Oosterhout, additional field work on the Great Tit.
7. Liesbosch, additional field work on the Great Tit.
8–9. Zuidelijk and Oostelijk Flevoland, newly reclaimed Zuiderzeepolders scene of the field work of the Department of Distributional Ecology.
10. *Voorne's Duin* (dunes of Voorne), with the Biological Station *Weevers' Duin*, site of the Department of Botanical Ecology.
11. Dunes of Goeree, additional field work of the Department of Botanical Ecology.

3. Population Ecology

3.1. POPULATION DYNAMICS OF THE GREAT TIT, *Parus major*

3.1.1. *Mortality, dispersal, and moult in* 1971/1972 (J. H. van Balen)

Since 1967 the Great Tit population in the Hoge Veluwe study area has been studied on the basis of captures and recaptures at regular intervals during the summer, autumn, and winter. This method permits

assessment of the changes in numbers from one breeding season to the next. These changes are due to immigration, emigration, and mortality, which cannot be distinguished in all cases. In addition, the condition of the tits is studied on the basis of the body weight and the moult score.

Numerical decreases usually occur in the summer, mainly due to mortality and emigration of yearlings, and in the autumn, probably due to emigration. Immigration into the study area is common in July, September, and March, but the numerical importance varies strongly from year to year.

This study will be continued in 1972/1973, after which the results will be analyzed.

3.1.2. *The tit irruption in the autumn of* 1971 (J. H. van Balen, B. J. Speek)

Several *Parus* species do not migrate regularly, at least in the northwestern part of Europe, but instead show irruptions. These irruptions vary in strength, probably due to varying numbers in the area of origin and to a varying proportion of migrants within the population.

In The Netherlands tit irruptions are infrequent, but a large irruption of Great, Blue, and Coal Tits (*Parus major*, *P. caeruleus*, and *P. ater*) was observed during the autumn of 1971. Since immigration and emigration are important factors in population dynamics, it was decided to collect information on this tit irruption from several sources, and to determine, if possible, whether there was a relationship between the extent of migration and certain environmental variables.

The many observations and ringing captures (especially of foreign-ringed birds) indicated that the number of immigrants was larger than in any year since 1961. The first immigrants were observed toward the end of September. The irruption ended early in November, but foreign-ringed tits were observed until March of 1972. Most of the irruption was confined to the northern, western, and central parts of the country. A considerable proportion of the immigrants spent the winter in The Netherlands. Some of the tits ringed during the irruption period were recovered or recaptured in western Belgium and southeastern England during the following winter. Other recoveries showed that some of the birds migrated in a northeasternly direction during March.

The foreign-ringed tits originated from northern Germany, northern Poland, and the Estonian, Latvian, and Lithuanian S.S.R. The birds from the Baltic area arrived earlier than the German tits. The recoveries of German tits enabled us to calculate the standard direction which proved to be 268° (nearly due West). The average distance covered by the Baltic tits per day amounted to about 40 km.

The proportion of females among the migrating Great Tits was fairly high, and the proportion of yearlings (of the Great and Blue Tit) was also larger than expected.

Irruptions of Great Tits are usually associated with a high population density and/or poor feeding conditions in the area of origin. The influence of these environmental factors on the 1971 irruption is obscure, because the conditions in the area of origin are unknown. Although in The Netherlands the 1971 breeding population was high in most areas and the beech crop was very poor, the movements of the Dutch Great and Blue Tits were only slightly more frequent than in other years.

3.1.3. *Factors affecting clutch-size* (J. H. van Balen, L. A. Kajim)

The experiments on the relationship between the width of the nesting hole and the size of the clutch (discussed in detail in the 1971 Progress Report) were continued. Again, in half of the nestboxes in Liesbosch the internal dimensions were reduced to 6×6 cm (normal dimensions: 9×12 cm). The results agreed closely with those obtained in 1971. Both Great and Blue Tits preferred the normal-sized to the small nestboxes and in both species the clutch-size was significantly smaller in the small boxes.

This demonstration of the effect of the dimensions of the nesting cavity on clutch-size raises two questions. The first is, what stimuli induce the female to adjust the number of eggs to the conditions offered by the nesting cavity? The second concerns the ultimate aspect of the relationship, which possibly lies in the thermo-regulation of the brood, i.e. in the risk of reaching a hyperthermous state, which must be great for a large brood in a small cavity.

The above-mentioned observations led to an attempt to determine the stage of the reproductive cycle in which the clutch-size can be modified by external stimuli. This aspect was studied in 1971 (see Progress Report for 1971) by the addition and removal of eggs to and from clutches at different stages of the laying cycle. These experiments were continued in 1972 by removing eggs from the second or third day of laying and by adding eggs on one of these two days. In general, changes made on the second day of laying induced the tits to modify the number of eggs laid, whereas changes made on the third laying day had no effect. Compensation for the removal or addition of eggs was not complete.

3.1.4. *Experiments on the regulation of numbers in the Vlieland population* (J. H. van Balen, H. M. van Eck)

During several successive years, the regulation of numbers in the Great Tit has been studied by reducing the reproduction in the Vlieland population and comparing the annual survival of adult and juvenile birds in normal and experimental years. These experiments resulted in an increased survival of the juveniles and, even more so, of the adults (KLUYVER 1971), due to reduced competition in the period between the fledging of the young and December.

In another type of experiment, started in 1970, the number of adults was reduced in the summer, to study the effect on the annual survival

of adults and juveniles. For practical reasons, the adults had to be taken when they were attending their second broods. This resulted in a small reduction of the number of fledged young, which hindered the interpretation of the results to some extent.

Because the possibilities for catching adults in summer vary with the proportion of late broods, the proportion of adults taken varied strongly in the three experimental years (1970: 50%, 1971: 35%, 1972: 13%). For the first two of these years the annual survival rates are known. In 1970/1971 the survival of both adults and juveniles was higher than the survival rates obtained in the earlier experiments, in which the reproduction was reduced. In the second year the survival rates were about equal to those of the earlier experiments. Clearly, these experiments will have to be continued for some time before valid conclusions can be drawn.

3.1.5. *Observations and experiments on the ecology of the Great Tit* (H. N. Kluyver)

The investigation on the survival of young Great Tits after removal of the male parent was started in connection with LACK's (1954, p. 31) hypothesis that clutch-size in birds has been adapted by natural selection to correspond with the largest number of young for which on average the parents can provide enough food.

We took away the father of a number of broods at various ages of the nestlings, and determined the average number of young that left the nest. This number was not significantly lower than for broods fed by both parents, which means that the female was able to raise the young up to the time of fledging. The percentage of recoveries of ringed young of the half-orphaned broods was, however, significantly lower than for normal broods, most probably because the former lacked an adequate amount of reserve fat.

Males of these broods released elsewhere either remained in the new area or returned to their original breeding place. Some indications were found that males aged one year were more apt to return to their original area than were older males, which tended to stay where they were released.

The survival of young Great Tits in relation to nest infection by the parasitic fly *Protocalliphora azurea* Fall was also studied. A moderate infestation of these parasite larvae does not lead to a decrease in the number of young leaving the nest, but the percentage of recoveries of ringed young is lower.

Our provisional conclusion with respect to the influence of the loss of the male parent and parasitization is that they have about the same effect on the young tits: neither causes any decrease of the survival in the nest, but both lead to higher mortality after fledging. In relation to population dynamics they may only be responsible for less overpopulation in the summer and thus in turn for reduced competition in subsequent

months, which would increase the survival of the remaining young (see KLUYVER 1971).

(The above section is a summary of a paper to be published in *Ardea*.)

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3.1.6. *Parasitism by Protocalliphora spp.* (C. W. Eshuis-van der Voet)

The study of the influence of larvae of *Protocalliphora* on the haemoglobin level in the blood of nestling Great Tits was continued.

To estimate the haemoglobin level of the blood of uninfected nestlings, we tried to take blood samples from birds aged 3, 6, 9, 12, and 15 days, but sampling proved to be impossible in 3- and 6-day-old birds. Data for the older nestlings are shown in Table 1.

Table 1. Haemoglobin level (g/100 ml) in blood of Great Tit nestlings.

Age of nestlings (in days)	Mean	Range
9	8.98	7.45–10.70
12	10.23	7.49–13.22
15	11.89	8.30–14.60

Since the published values for adult pigeons and chickens lie as high as about 14.00–16.00 g Hb/100 ml, it seems possible that the haemoglobin level of the nestling tits increases to a comparable level after 15th day.

Kluyver (in press) found a decrease in fledgling survival at increasing numbers of *Protocalliphora* in the nest. As mentioned in earlier reports, we found no effect in infected nestlings on the basis of 15th-day body weight, 15th-day wing length, or duration of the nestling period, as possible parameters for fitness of nestlings. The 15th-day haemoglobin level was usually lower in infected than in uninfected nests.

The extent of an infection on the 15th day can be defined more exactly on the basis of the weight of the larvae per nestling (neglecting number and age of the larvae), but even then no influence of infection on the 15th-day body weight, 15th-day wing length, and duration of nestling period was detected. The slight indications for such an influence mentioned in the preceding report were not confirmed by the 1972 data. However, the haemoglobin level decreases rather steadily at increasing weight of larvae per nestling (Table 2).

Table 2. Relationship between haemoglobin level (in g/100 ml blood) of Great Tit nestlings and weight of *Protocalliphora* larvae.

Weight of larvae per nestling (in mg)	Mean haemoglobin level (g Hb/100)	Number of nests sampled
0	11.89	23
1– 100	11.94	17
101– 200	11.32	2
201– 300	10.81	1
301– 400	—	0
401– 500	9.47	1
501– 800	—	0
801– 900	7.79	1
901–1000	8.14	1

Probably due to the rainy weather in the 1972 season, there were few severely infected nests.

Data on larval movement in the nest material during a 24-hour period were obtained by giving the tits a wadding nest composed of clearly recognizable layers. When sampling these nests, we registered the time at which larvae were found in a certain layer. So far, data have been collected this way for the interval between 7.00 am and 17.00 pm. In this interval the highest number of larvae (about 70–90%) was already found in the top layers of the nest, at a short distance from the nestlings. No relationship was found between the body weight of the larvae and their location in the nest material.

In a number of larvae of different weight the meal size was determined by weighing individuals immediately before and after a meal. The smallest larvae (<0.1 mg) may suck an amount of blood up to about 3–4 times their body weight, the larger ones (20.0–80.0 mg) up to about 1½ times their own weight.

The variation in larval body weight within one nest is often considerable. In many cases this variation was already due to the large amount consumed, from which it may be assumed that some larvae had had one meal more, others one meal less, than most of the others. In other cases nests were apparently infected more than once.

The nest layers chosen for pupation were also registered, and the data for nests still occupied by nestlings were compared with those of nests where nestlings had already fledged at pupation time. When nestlings were still present, only a few pupae were found in the top layers of the nest; after fledging of the birds the pupae were more evenly distributed over the nest. The larvae evidently prefer a relatively low temperature for pupation, which is in accordance with our earlier conclusion that survival of pupae is about 100% when the temperature is lower than about 27° C. At rising temperatures the percentage of surviving pupae decreases rapidly, mortality being 100% at about 35° C. Near the nestlings,

i.e. in the top layers of the nest, the temperature becomes as high as about 25–35 °C; in the bottom layers of an occupied nest the temperature is lower, usually lying at about 15–25 °C (data from Mertens).

3.2. A MODEL FOR THE PREDICTION OF HEAT LOSS OF GREAT TIT BROODS (J. A. L. Mertens)

The eco-physiological work in 1972 mainly concerned the development of a physical model for the prediction of heat loss of broods. In addition, water loss measurements were carried out on Great Tit broods of various size at different temperatures to obtain information on the relative heat loss due to water evaporation.

For the heat loss model only the non-evaporation heat loss was taken into consideration. Non-evaporative heat loss occurs in three ways, i.e. by conduction, radiation, and convection. All of these three models of heat loss can be regarded as being proportional to the temperature difference between nestling skin and the inner wall of the nestbox. The proportionality constant is called the conductance.

These three conductances and the relationship between brood weight and over-all conductance are discussed below.

3.2.1. *Radiation conductance (Crad)*

The radiation conductance per cm^2 (α rad) is determined by the absolute temperature and emissivity of the inner surface of the wall of the nestbox and the emissivity of the nestling skin.

By approximation:

$$\alpha \text{ rad} = 4 \cdot \sigma \cdot \varepsilon_b \cdot \varepsilon_{nk} \cdot T_{nk} \qquad [\text{gcal/hr. cm}^2\text{. °C}]$$

where σ is the Stefan Boltzmann constant, T_{nk} is the absolute temperature of the inner surface of the wall of the nestbox, and ε_b and ε_{nk} the emissivities of skin and inner surface of the wall, respectively (both assumed to be about 1).

Therefore, in the heat loss model the equation

$$\alpha \text{ rad} = 4 \times 0.49 \times 10^{-8} \times T_{nk}^3 \qquad [\text{gcal/hr. cm}^2\text{. °C}]$$

was used.

The radiating surface amounts to about 80% of the uncovered brood surface. The surface (S) of a brood can be computed from its weight (W) by applying the equation $S = 5.004\ W^{2/3}$ cm^2, if we regard the shape of the brood as a sphere. About half of this surface will be uncovered, so the radiating surface will be 40% of the total surface.

The radiation conductance will therefore be:

$$C \text{ rad} = 2.0016\ W^{2/3} \times \alpha \text{ rad} \qquad [\text{gcal/hr. °C}]$$

3.2.2. *Convection conductance (Cconv)*

Heat loss by convection is effected by air movement at the uncovered upper surface of the brood, the air layer in contact with the warm skin being warmed, causing it to rise and be replaced by heavier colder air. This results in a convection component in the heat loss having a convection conductance per cm^2 of

$$\alpha \text{ conv} = 0.4716 \sqrt[4]{\frac{t_b - t_{nk}}{\sqrt[3]{0.79\,\pi^2\,W}}} \qquad [\text{gcal/hr. cm}^2\text{. °C}]$$

where t_b denotes the skin temperature, t_{nk} the air temperature near the inner surface of the nestbox wall (both in °C), and W the weight of the brood (in g).

Convective heat loss takes place through the uncovered upper surface of the brood, which represents about half the total surface.

Thus the convection conductance is:

$$C \text{ conv} = 2.502\ W^{2/3} \times \alpha \text{ conv} \qquad [\text{gcal/hr. °C}]$$

3.2.3. *Conduction conductance (Ccond)*

Heat loss by conduction results from the warming of still air by adjacent warmer still-air layers.

The conduction conductance was approximated by performing computations on a model of the brood-plus-nestbox system. The brood was assumed to have a spherical shape and the heat resistance of thin concentric layers (1 mm thick) of air and of nest material was computed, using heat conductivity values of 2.15 and 3.01 gcal/hr. cm^2. (°C/mm) for the air and nest material, respectively.

The conduction conductance is the reciprocal value of the sum of heat resistance of the concentric layers between the brood surface and the inner side of the nestbox.

In this way the equation:

$$C \text{ cond} = 1.056\ W^{0.653} \qquad [\text{gcal/hr. °C}]$$

was found for the conduction conductance.

3.2.4. *The relation between brood weight and over-all conductance*

The relation between W and the total conductance of the system proved to be more or less linear, and can be described by:

$$Cnk = 2.994\ W^{0.613} \qquad [\text{gcal/hr. °C}]$$

which is in good agreement with experimental results obtained in other years (see 1970 Progress Report).

3.3. ECOLOGY OF THE COLLARED DOVE, *Streptopelia decaocto* (J. H. van Balen, D. Westra)

For the third year in succession the productivity of the Collared Dove population near Ermelo was studied. For this purpose all nests in a 60-ha area were inspected at least weekly from March until October.

The proportion of nests started in March was considerably higher than in preceding years. This points to an earlier start of laying in part of the population. Otherwise, the distribution of the first-egg dates in 1971 and 1972 was similar.

The 29 pairs in the study area produced 116 clutches, i.e. 4 clutches per pair. As in 1971, the mean clutch-size amounted to 1.96 eggs and did not vary throughout the season. Egg losses were slightly higher for the clutches started in March than for the remaining clutches. Nestling losses decreased strongly throughout the season, probably due to decreased predation. The productivity per clutch again varied strongly, and showed a peak for nests started from June to September. The earlier finding that the period in which the most clutches are started is also the most productive period, was not confirmed this year. We hope to continue this study in 1973.

The over-all productivity per pair was estimated at 2.6 fledglings per pair, which is appreciably lower than the value found in 1971 (4.0 young per pair).

3.4. ECOLOGY OF THE COOT, *Fulica atra* (J. H. van Balen, J. Visser)

The field study on the Westereinderlakes near Aalsmeer was continued. The main object of this study is to determine the relationship between breeding density, clutch-size, and laying date. This is done by comparing data from three habitats with widely differing breeding densities and by studying annual differences within the habitats. Some preliminary results have already been reported (Progress Report, 1970).

Some of the work is concerned with ringing, i.e. for a study of the age composition of the breeding population. Numerous data on body weight and wing length have been collected (which can now be handled by machine sorting). Wing length is an important parameter for sex determination. Females have shorter wings than males, but the area of overlap is considerable. Yearling Coots have shorter wings than adults. Among the adult birds sex difference in wing length is such that the frequency distributions overlap only to a small extent.

Coots born or breeding in the study area have significantly longer wings than immigrant Coots. It is not known whether this is due to local differences or geographical trends in wing length.

Abrasion leads to a decrease in wing length during May-June as compared with the September-April period. Significant annual differences in wing length were found, the highest value occurring in 1967/1968 and the lowest in 1969/1970. The temperature during the moulting period may play a role here.

4. Bird migration

4.1. SPONTANEOUS MIGRATION ACTIVITY OF CHAFFINCHES IN THE KRAMER CAGE (A. C. Perdeck)

4.1.1. *Choice of direction and directiveness throughout the year*

The 1969 and 1971 Progress Reports included some preliminary results concerning the problem of whether the choice of direction shows any annual pattern.

In the spring and autumn the chaffinches in the Kramer cage showed directional choices agreeing very well with the directiveness to be expected from field observations (NE and SW, respectively). In the summer and winter there was no significant concentration in a certain direction, and no consistent trend of the mean monthly directions could be detected.

The latter conclusion was analyzed again by ranking the monthly mean direction given in the 1971 report. These directions are given in Table 3 under selection I. NW (310°) was chosen as starting-point, and the monthly mean directions are ranked according to their clockwise increase of angle from this point onward. The ranking of selection I suggests that the monthly mean direction shifts gradually from NW in January to SE in July and suddenly to NW in August, after which it turns the other way round to reach S in December. To test this impression another selection of the material was made (selection II, Table 3). The hourly observations in which the birds were not significantly directed, were added to the observations of selection I, as well as the monthly means of not-significantly directed birds.

Table 3. Mean monthly directions of Chaffinches in the Kramer cage.

Month	Selection I		Selection II	
	Mean direction	Rank	Mean direction	Rank
January	310° (NW)	1	315° (NW)	1
February	356° (N)	2	356° (N)	2
March	79° (E by N)	5	85° (E by N)	5
April	41° (NE)	3	31° (NE by N)	4
May	46° (NE)	4	15° (N by E)	3
June	87° (E)	6	89° (E)	6
July	118° (SE by S)	7	199° (SSW)	8
August	286° (W by N)	12	216° (SW by S)	9
September	229° (SW)	11	232° (SW by S)	11
October	224° (SW)	10	233° (SW)	10
November	218° (SW by S)	9	265° (W)	12
December	105° (S)	8	152° (SSE)	7

Since it was found that the directional choice later in the day (sun azimuth $>225°$) is not appreciably different from the choice earlier in the day, the hourly observations later in the day were also included.

Observations during which the sun could not be precisely located were excluded; this was done to eliminate disturbance due to lack of directional choice resulting from invisibility of the sun. The directions of selection II were ranked in the same way as those of selection I. The ranking of selection II suggests a gradual turn from NW in January to E in June and to W in October.

The least complicated hypothesis based on these observations is that over the period of a year the preferred direction turns 360° clockwise. This hypothesis can be tested with Kendall's rank correlation test against the alternative hypothesis that no turning occurs. The latter hypothesis has to be discarded ($P < 0.0015$ for both selections; tested one-sided).

It might be that the shift suggested by the material is caused mainly by the directional preferences in the migration months. This is not the case, however, because the hypothesis that no turning occurs must also be discarded when the six migration months (March-May; September-November) are excluded ($P < 0.05$).

To summarize, it may be stated that there is some evidence that directional preferences turn 360° clockwise over the period of a year such that in the migration months these preferences are in the migration directions.

To detect oscillations of directiveness throughout the year, the mean monthly vector lengths from selection II (including not-significantly directed observation hours) given in Table 4 were analyzed. The circular runs test (BATCHELET, 1965) can be used to detect oscillations of the vector length throughout the year. No significant oscillation was found.

Table 4. Mean monthly directiveness of Chaffinches in the Kramer cage.

Month	Mean vector length	Above (+) or below (−) median (43)	Number of individuals
January	38	−	8
February	64	+	7
March	34	−	8
April	47	+	14
May	20	−	12
June	49	+	8
July	50	+	6
August	15	−	8
September	72	+	7
October	62	+	11
November	37	−	9
December	16	−	11

It has been stated above that there is no large difference in the directional choice throughout the day. This conclusion is based on an analysis of the directions found in three periods of the day characterized

by the azimuth of the sun. This analysis was carried out separately for the winter (December-February), spring (March-April), summer (June-August), and autumn (September-November). The results are given in Table 5.

Table 5. Mean direction throughout the day of Chaffinches in the Kramer cage. (Number of individuals between parentheses.)

	Azimuth		
	0°–135°	135°–225°	>225°
Winter	82° (3) E by N	309° (9) NW by W	215° (5) WSW
Spring	38° (19) NE by N	67° (12) ENE	22° (11) NNE
Summer	93° (11) E	136° (9) SE	158° (10) SSE
Autumn	188° (10) S by W	238° (18) SW by W	270° (7) W

In the summer and winter there is a tendency to turn in the same direction as the sun during the day. This might be due to aberrant behaviour in the Kramer cage, which possibly becomes more apparent in the periods when the birds are more or less directed to the sun. Possibly, they then use a simpler orientation mechanism in which the shift in the sun's azimuth in the course of the day is not compensated for.

4.1.2. *Experiments on the influence of day length on orientation*

It might be supposed that the directional choice in the migration periods is determined by the shift in day-night rhythm throughout the year. If this supposition is true, it must be possible experimentally to let the birds choose the autumn direction in spring (and the spring direction in autumn) by keeping them for a considerable time in a day-night rhythm that changes in the normal way but is antedated by six months. Such experiments were described in the 1971 report and some preliminary results were also given. These experiments have been continued and the results are given in Table 6.

Table 6 includes only individuals showing a significant directiveness during the tests. The data indicate that these birds did not concentrate in a given direction. Consequently, the total mean directions are not significant and the results of these experiments are inconclusive. The high scatter in the directional choices might easily be due to the experimental situation. It was necessary to bring the birds back and forth from the light-controled room to the Kramer cage, which had to be done by hand twice a day, and this may have been too great a disturbance. Experiments are being planned to overcome this difficulty.

4.2. EXPERIMENTS ON DISCRIMINATION BY THE STARLING BETWEEN GEOGRAPHICAL LOCATIONS (A. J. Cavé)

The goal and set-up of these experiments are discussed in detail in the

Table 6. Directions of Chaffinches in the Kramer cage. Day-night rhythm antedated 6 months.

ind. nr.	Tested in spring direction	ind. nr.	Tested in autumn direction
1	73° E	12	89° E
2	132° SE	13	105° E
3	178° S	2	179° S
4	191° S	14	122° SE
5	201° S	15	206° SW
6	219° SW	16	239° SW
7	266° W	17	359° N
8	308° NW		
9	332° NW		
10	354° N		
Total mean	251° W		145° (SE)

1970 Progress Report (pp. 59–60). The study is being continued with a larger number of birds. At this moment eight birds are in use.

The results given below must be considered highly tentative. A detailed analysis is in progress.

In the 1971 report it was concluded that adult female Starlings are able to learn to discriminate between two training places (Monster and Gimbte) if they have a full view of the surrounding landscape and can see the sun. Later tests have confirmed this conclusion.

It was also reported (1971) that some of the birds that were able to discriminate between the two places when the sun was visible failed to do so under total overcast, but later experiments showed that most birds are able to discriminate between two places under the latter conditions. This means that in addition to the sun most birds use cues given by familiar landmarks or other cues not yet known.

Five birds were tested in a different landscape situated at a short distance from the training place. Due to circumstances, this was done only in Gimbte and each bird was tested only once, under sunny conditions. The tests of two of these birds are considered unreliable; the data on the other three show some preference for the correct perch (correct 49 times; wrong 28 times). The results suggest that these birds did not need familiar landmarks to recognize "Gimbte".

To eliminate the effects of landmarks, we screened off the landscape at both locations in two ways, i.e. with

a. a 2 m square canvas screen, and

b. a circular aluminium screen (inner surface dull-black) 1 meter in diameter.

With both arrangements the cage was placed in the centre. The birds had no view of the landscape during either transport or placement in the experimental situation. The screen had the same height in both lo-

cations, and the birds were trained within the screen. Even after prolonged training the birds did not learn the difference between the two places. On the contrary, they showed a preference for the wrong perch during testing at one of the two places (4 birds; total for Monster: 381 times correct, 606 times wrong; total for Gimbte: 406 times correct, 390 times wrong). Since the last training before testing always took place at the other locality, the preference for the wrong perch is understandable, but it is not clear why this phenomenon only occurred at one of the two places.

The results of the earlier experiments suggested that at least some of the birds discriminated between the two places without using familiar landmarks aided by the position of the sun. The negative results of the present experiments with the landscape screened off indicate, however, that the birds need a landscape.

To understand this we must consider the possible role of the sun in more detail. Let us suppose that the birds use the height of the sun and/or its movement along the sky. To measure the height of the sun the horizontal plane at eye-level is needed, and observation of horizontal movements requires reference points at considerable distances. Since the screens were quite close to the cage, the angle between the sun and reference points on the screen would vary with each movement of the bird.

If this reasoning is correct, hiding of the landscape means that not only familiar landmarks are screened off but also the reference points needed to observe the ecliptic of the sun. One way to reduce this effect of a screen would be to fix the bird in the centre of the screen. This, however, can be expected to influence the bird's behaviour strongly. Another way would be to test the birds systematically, with and without visibility of the sun, in other landscapes at localities near the training place. Such experiments are being carried out.

5. Department of Distributional Ecology

5.1. INTRODUCTION

Large recently reclaimed areas, such as the new IJsselmeer polders, offer excellent opportunities to study dispersal in terrestrial plants and animals. The Department of Distributional Ecology started its investigations with a detailed study of the colonization of the Oost Flevoland polder (reclaimed 1957) by a few insect species and the mole *(Talpa europaea)*. When the Zuid Flevoland polder became dry in 1968 it was considered useful to widen the scope of the investigations by taking more species into account, because this could offer a basis for more intensive study of promising problems at a later stage.

Three lines of investigation were started in Zuid Flevoland, each covering a different group of organisms. The colonization of the muddy

soil by vascular plants has been studied along with the analysis of the development of the vegetations of pioneer plants. The pioneer vegetations can still be studied in restricted parts of the polder, but in most areas the vegetation succession soon culminated in dense reed fields with little admixture of other plant species. Currently, these reed vegetations show signs of deterioration, and this development and its causes are being intensively studied in an experimental reed field. This experiment is reported for the first time here.

Another line of investigation concerns phytophagous insects living on reed and willows, most of which colonized the polder within a few years. The population development of one of these insects, the Noctuid moth *Archanara geminipuncta*, and its interaction with the reed vegetation is being followed in the experimental field mentioned above. Divergent behaviour is shown by the aphid *Hyalopterus pruni*, whose yearly host-plant alternation coincides with an annual migration from the "old land" to the polder. This special behaviour will be treated below in some detail.

A third line of investigation comprises the study of Carabid beetles and other ground-dwelling Arthropods that can be caught in funnel traps. Preliminary results and information on methods are given in this report.

After evaluation of the results obtained so far, it should be possible to identify and describe some main strategies of colonization by plants and animals and at the same time to formulate theories concerning the means and the function of dispersal that can be tested in later research.

5.2. A FIELD EXPERIMENT ON THE DEVELOPMENT OF REED VEGETATION (J. van der Toorn, J. H. Mook)

After drainage of an IJsselmeer polder a rapid succession starts on the muddy soil with an early pioneer vegetation and continues until the stage of a closed stand of Common Reed *(Phragmites australis)* is reached. To accelerate this development, most of the Oostelijk and Zuidelijk Flevoland polders was sown with reed seed (by the Polder Authority). The reed attains its maximal vitality three of four years after drainage. Observations in Oostelijk Flevoland (HEMMINGA and VAN DER TOORN, 1970) have shown that from then on reed growth declines, especially in dry places, and other species establish themselves in the vegetation.

A number of simultaneous changes in the environment of reed may induce this development. The most important are thought to be the following: the drying-up of the soil, the strong decrease in the mineral nitrogen content of the soil (VAN SCHREVEN, 1965), and the heavy infestation of the reed by larvae of noctuid moths, especially *Archanara geminipuncta* (see 1971 Progress Report).

To investigate this problem, we thankfully accepted the opportunity to work on an experimental reed field set up in Zuid Flevoland by the IJsselmeerpolders Development Authority. In this field wet and dry parts were created in 1971, making it possible to study the influence of the

water table. Part of the vegetation is burnt off in the winter, as a result of which the overwintering stages of most insects (including *Archanara geminipuncta*) are eliminated. Because *Archanara* overwinters in the egg stage and the larvae do not migrate, these burnt vegetations are free of damage by this insect during the following summer.

In 1972, regular observations were started on the growth of the exposed parts of Common Reed in four environmental situations, viz. under wet and dry conditions, both burnt and unburnt, and the underground parts will be sampled each winter in the same four vegetations. The populations of some insects and the damage they cause to the reed will also be studied. Another aspect that will be followed is the species composition of the vegetation. *Epilobium hirsutum* is becoming especially important in the unburnt vegetation.

The influence of the depletion of nitrogen in the soil will be followed in experiments with fertilizers.

The data collected in 1972 point to important effects of both water table and moth larvae. The observations will be continued for several years for the investigation of long-term effects. Experiments will be carried out in the greenhouse and in the field, near the laboratory, to study the details of the processes involved.

References

HEMMINGA, M. A. and J. VAN DER TOORN, Toepassing van riet in de IJsselmeerpolders, Flevoberichten, Zwolle **73**, 1–31 (1970).

SCHREVEN, D. A. VAN, Stikstof omzettingen in jonge IJsselmeer gronden, van Zee tot Land, Zwolle **41**, 1–74 (1965).

5.3. THE IMMIGRATION OF THE APHID *Hyalopterus pruni* IN ZUIDELIJK FLEVOLAND (J. H. Mook, J. Wiegers)

The Mealy Plum Aphid (*Hyalopterus pruni*) is an especially suitable object for the study of immigration in the IJsselmeer polders because this species hibernates exclusively on Plums (*Prunus domestica* and *P. insititia*) and on Blackthorn (*Prunus spinosa*), which do not occur in a new polder until woods and shelter belts along roads are planted.

Common Reed (*Phragmites australis*) is a secondary host plant for this aphid, and the reed in the Zuid Flevoland polder is infested each year at the end of May or in June by alate virginoparae originating from *Prunus* in the surrounding older land.

In the 1968 and 1969 Progress Reports short accounts were given of the occurrence of *Hyalopterus* on reed along a transect running in a SW-NE direction through the centre of Zuid Flevoland. In both years there was a marked decline in the infestation of the reed, beginning near the SW dike and proceeding in a NE direction. This decline could hardly be the effect of distance alone, but an adequate hypothesis to explain it could not be given.

In 1971 and 1972, migrating aphids were caught with sticky traps, again along a SW-NE transect but more frequently (owing to the greater accessibility provided by a recently constructed road) and in a different pattern with respect to the distribution of the reed. In 1968 and 1969, just after drainage of the polder (spring of 1968), the reed was evenly distributed along the transect, whereas in 1971 and 1972 no reed was present in the parts of the transect nearest to the dikes, where reclamation was proceeding. In both of the latter years the aphid traps were examined every three or four days and the catches were found to be highest in the SW or the NE part of the transect, depending on the direction of the prevailing wind at the time. This is perhaps not surprising, but we also got the strong impression that the decline started not at the edge of the polder but at the edge of the reed field. The data are insufficient to fully substantiate this impression, and therefore the observations will be continued in 1973.

It is thought that a decrease in the number of trapped aphids from the edge to the centre of the reed field could be due to the influence of reed colour on the landing behaviour of *Hyalopterus*, shown experimentally by MOERICKE (1969), as a result of which most *Hyalopterus* landed near the edge of the reed field and relatively few remained to be caught further away.

Reference

MOERICKE, V., Host plant specific colour behaviour by Hyalopterus pruni (Aphididae). Ent. exp. appl. 12, 524–534 (1969).

5.4. COLONIZATION OF THE IJSSELMEER POLDERS BY CERTAIN INSECT GROUPS AND PLANTS (J. Haeck, R. Hengeveld)

The ultimate problem of our study concerned the dispersal and establishment of organisms in relation to environmental conditions, which involves two lines of research, one concentrating mainly on dispersal and the other on the preferred environmental conditions of the species in question. These lines cannot be separated too rigorously; it may, for instance, depend on the conditions whether or not an animal is activated.

We wished to give special attention to the dispersal capabilities of a wide variety of species of *Carabidae*, *Lycosidae*, and other epedaphic groups, and this year we also took plants into consideration in this respect. These capacities can only be studied, however, if they can be measured as one of many factors influencing the occurrence of particular species in a certain area. To estimate the weight of the dispersal capabilities relative to environmental factors, we performed a principal component analysis on the data concerning the numbers of Carabid beetles caught along a sampling transect in Zuidelijk Flevoland, covering a two-year period (1970 and 1971). The results indicated that the environmental factors prevail over the dispersal capabilities or even account for all the variance.

Pterostichus coerulescens
Noord oost
Polder
Zuidelijk
Flevoland
Oostelijk
Flevoland
1 4 2
1
1
2
2
53
11
30
103
0 1 2 3 4 5 km.

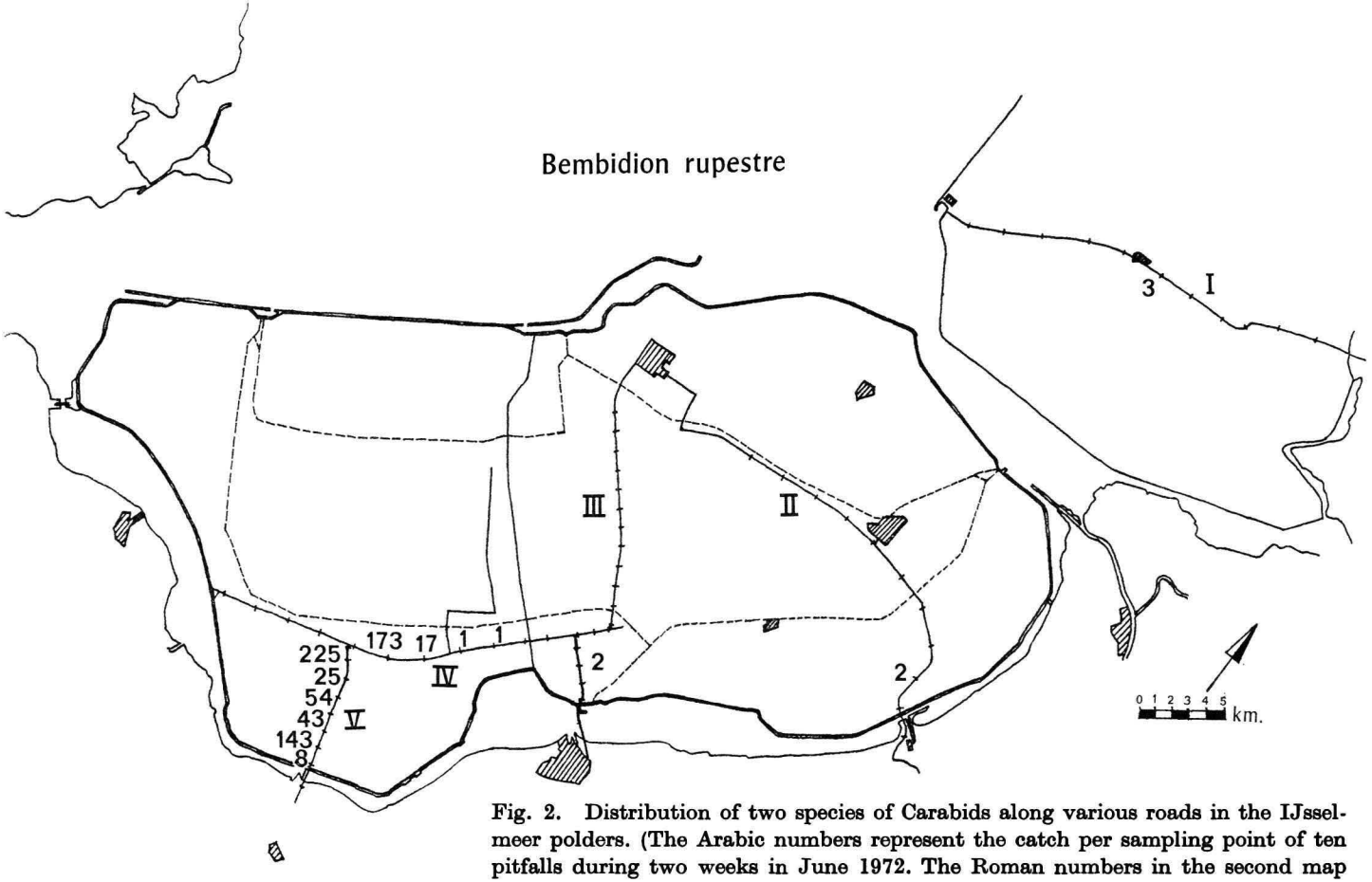

Fig. 2. Distribution of two species of Carabids along various roads in the IJsselmeer polders. (The Arabic numbers represent the catch per sampling point of ten pitfalls during two weeks in June 1972. The Roman numbers in the second map refer to the road-numbers in Table 7).

In 1972, in three areas (Fig. 2) we sampled the above-mentioned groups along roads running at right angles to the coast. These roads were built at different times: the Noordoost polder was drained in 1941, Oostelijk Flevoland in 1957, and Zuidelijk Flevoland in 1968. Per road, the sampling points were located at increasing distances from the mainland. The vegetation on the verges was assumed to be uninterrupted and more or less homogeneous. To check this assumption we described the species composition of the vegetation at the sampling sites, which revealed that the composition of the vegetation differs from one polder to another according to age. It was also found that the number of plant species per road decreases with increasing distance from the old land. Therefore, as far as the composition of the vegetation is concerned, the sampled verges may not be considered homogeneous.

Comparison of preliminary data on carabid beetles and wolf spiders gave a picture differing from that of the plants. Although some species occur exclusively at a given place, suggesting an age effect, the distribution can as a rule be entirely explained by conditions prevailing locally, i.e. vegetational structure, soil conditions, or distance from a grove. Table 7 shows a trend from left to right, parallelling the ages of the roads, in a restricted sample of all the species in question. Fig. 2 gives an example of two species from Table 7. Soil samples from the various sites showed that *Pterostichus coerulescens* occurs exclusively in places with sandy soil. *Bembidion rupestre* is known from the literature to occur in very open vegetation on moist soils, precisely the conditions under which it was

Table 7. Preliminary data concerning catches of Carabidae, Lycosidae, and Isopoda along various roads in the IJsselmeer polders (see Fig. 2).

Species	Percentage occurrence on road nr.*						Total number caught
	0	I	II	III	IV	V	
Philoscia muscorum (Is.) **	2	98					527
Amara communis (C.)	1	40	57	1	1		1,425
Pterostichus coerulescens (C.)		25	70	3	2		286
Pterostichus strenuus (C.)		27	9	60	3	1	1,300
Pardosa prativaga (L.)		12	31	51	7		1,078
Pardosa amentata (L.)		29	3	43	25	1	1,110
Anisodactylus binotatus (C.)	1	4	3	68	3	1	1,215
Pterostichus cupreus (C.)	1	5	6	69	17	2	1,452
Pardosa monticola (L.)			2	49	48		1,032
Pirata piraticus (L.)	1	1		15	68	15	572
Bembidion ustulatum (C.)		4	1	22	17	56	1,397
Bembidion rupestre (C.)					28	71	697
							12,091

* Roads indicated (0 . . . V) in order of decreasing time since construction, 0 representing two sample plots on the "old land" and V the most recent road.
** Is. = Isopoda; C. = = Carabidae; L. Lycosidae.

PLATE I – A familiar scene in Arnhem: interior of a nest box with Great Tits.

PLATE II – A familiar scene in Voorne: interior of the dunes with signs of industry in the not very distant background.

found in the polders. It seems likely that after collecting more data on this point, we will have to shift the main emphasis of this research to the environmental side of the problem.

HAECK and REIMERINK (see 1971 Progress Report, pp. 85–87) have shown that of the relevant conditions the moisture level of the soil is the most important. The influence of this factor is thought to be exerted particularly on larval hibernation. This point may form one aspect of our future research, others being spatial patterns and their dynamics within an animal population and the genetic aspects of the development of various populations of known age.

6. Botanical Ecology (Weevers' Duin Biological Station)

6.1. THE MICROCLIMATE OF THE OPEN SHADOW (Ph. Stoutjesdijk)

In the 1971 Progress Report mention was made of the peculiar microclimate found at the north side of patches of shrubs and other obstacles such as steep hills and walls. That conditions are unusual in such places was first recognized with respect to the light climate, for which SEYBOLD (1936) created the term *Blauschatten*. A few of our data suffice to show that the light climate in the open shade is fundamentally different from that under a canopy or in the sun. The following Table shows the distribution over two wavelength ranges of the short-wave radiation received under a shrub canopy, along the northern edge of a shrub, and in the open. For the relative radiation intensities, photosynthetically active radiation in the sun (wavelength <700 nm) is taken as unity.

	Relative intensity of short-wave radiation		Wavelength interval of max. intensity	Net radiation
	<700 nm	>700 nm		
Open	1	1	500–600 nm	strongly positive
Under canopy	0.01	0.05	700–800 nm	weakly positive
Open shadow	0.15	0.05	400–500 nm	weakly negative

It is clear from this Table that there are considerable qualitative and quantitative differences between the short-wave radiation climates of the three localizations, and the same holds for the net (short-wave and long-wave) radiation exchange.

The surface temperature under a canopy is roughly the same as the ambient air temperature; in the open shadow it is much lower and in the exposed situation much higher. Dew formation is absent under a canopy; in the open shadow the dew persists and in the exposed situation it quickly disappears.

It is evident that in many respects the shadow fringe on the north side of an obstacle is not a mere transition zone but has specific characteristics. The open shadow effect was observed all the year round. As could be expected, it is weakest around the time of the summer solstice. Under our conditions, it is less well developed in the spring than in the autumn, because the shadow-casting shrubs are leafless in the spring. Biologically, the effect is probably most important in the late summer and early autumn, when there are prolonged periods of dry, still weather with a bright sky. Surface temperatures then often lie 6–10° C below the ambient air temperature. It is not yet completely clear how these low temperatures are maintained. The weakly negative radiation budget cannot be solely responsible. Thermal inertia and evaporation from the wet surface are very probably of equal importance.

Finally, the importance of the macro-climate is evident: the effect will be strongest where diffuse short-wave and heat radiation from the sky are weakest, i.e. when and where clear skies prevail and especially at higher elevations.

As was to be expected, preference for the open shadow situation is shown by hygrophilous mosses, in the dunes of Voorne by *Mnium undulatum* and *M. cuspidatum*. Dr. J. Barkman has informed me that in the mountains of Central Europe there are a considerable number of mosses and liverworts that seem to have an exclusive preference for open shadow. This would be in accordance with the above-mentioned dependence upon macro-climatic conditions.

6.2. THE STUDY OF THE ROOT-NODIAL SYMBIOSIS OF *Hippophaë rhamnoides* (P. A. I. Oremus)

The current work is divided into three parts:

1. a quantitative description of nodulation in the field,
2. germination experiments, and
3. inoculation experiments.

6.2.1. *Quantitative description of nodulation in the field*

The study was performed in a *Hippophaë* stand (2.50 m^2) situated along a paved path in Meyendel and containing 34 shrubs varying in age from 2 to 7 years and in height from about 0.50 to about 1.25 m, many of them connected by underground suckers. In a 1.50 m deep channel around the stand, the root system was carefully dug out. The location of each nodule was mapped, as well as the position of the roots (if possible). Most of the nodules occur 10–50 cm below the soil surface, as can be seen from Table 8.

6.2.2. *Germination experiments*

These experiments were carried out to study the temperature sensitivity of the seeds of *Hippophaë* and the sterilization effect of certain temper-

Table 8. Distribution of the nodules in the soil.

depth (cm)	number of nodules per 6 m²	depth (cm)	number of nodules per 6 m²
0–5	30	50–60	44
5–10	68	60–70	26
10–20	147	70–80	21
20–30	288	80–90	11
30–40	107	90–100	2
40–50	84	100–110	5
Total			833

atures. After a 2-hr temperature treatment, 40 air-dried seeds placed in petri dishes containing 30 cc 5% aqueous agar (20 seeds/dish) and held in the dark for 7 days at 28 °C. The germination percentages are shown in Table 9.

Table 9. Influence of a temperature treatment on *Hippophaë* seed germination, expressed as percentage of the total number of seeds.

Incubation period (days)	Temperature (°C)				
	20°	63°	67°	69°	71°
2	20	15	5	0	0
3	52.5	55	22.5	5	0
4	70	72.5	67.5	30	0
5	70	75	72.5	30	0
6	70	75	75	30	0
7	70	75	75	30	0

It is clear from these results that pretreatment at 69 °C and higher for 2 hours inhibits the germination of *Hippophaë* seed. So far, there are no indications that these temperature treatments also have a sterilizing effect. The proportion of infected seeds was the same in all of the experiments.

6.2.3. *Inoculation experiments*

6.2.3.1. Inoculation method

After precultivation (see 1971 Progress Report, pp. 94–96), 3 endophyte-free *Hippophaë* plants were placed in 200 cc erlemeyers provided with 100 ml N-free Crone solution, and a known quantity of a diluted nodule suspension was brought into each flask. Seven days later the roots were washed thoroughly and the plants placed in N-free Crone solution. After 3–4 weeks, the nodules formed on the roots were counted.

6.2.3.2. Influence of the age of the plants on nodulation

To study this influence, plants of different ages were inoculated at the same time with the same nodule suspension. This experiment was done in triplicate (Table 10).

Table 10. Influence of the age of the plants on nodulation.

Age of the plants (days)	Amount of nodule material (mg)	Number of nodules on three plants		
	100	2	19	37
	10	2	13	0
50	1	0	0	0
	0.1	0	0	0
	0.01	0	0	0
	100	0	3	3
	10	0	0	0
60	1	1	0	0
	0.1	0	0	0
	0.01	0	0	0
	100	6	5	3
	10	1	0	0
96	1	0	0	0
	0.1	0	0	0
	0.01	0	0	0
	100	0	1	0
	10	0	0	0
110	1	0	0	0
	0.1	0	0	0
	0.01	0	0	0

In plants older than 50 days nodulation decreased sharply, but in a second experiment 70-day-old plants showed very good nodulation (equalling that of the "50-day-plants" of experiment I), whereas 77-day-old plants were hardly nodulated (like the "110-day-plants" of experiment I).

The results show unequivocally that the age of the plant has an influence on nodule formation. It is not clear, however, what age is optimal for nodulation. The main reason for the divergent results of the two series of experiments is that the plants were cultivated in the greenhouse, where climatological conditions vary strongly during the year. Therefore, the age of the plants is not the best parameter for the condition of such plants at the moment of inoculation. These experiments will be repeated next year with plants cultivated in phytotrons to eliminate the variation in climatological conditions.

6.3. THE INFLUENCE OF TRAMPLING AND SOIL COMPACTNESS ON THE DISTRIBUTION OF SOME *Plantago* SPECIES (C. W. P. M. Blom)

6.3.1. *Introduction*

One of the subjects of the ecological research in the dune areas on Voorne and Goeree concerns the influence of recreation and grazing on

the vegetation. The dunes in these regions are under strong recreational pressure, and, in addition, grazing was started a number of years ago as a form of management. One effect of both recreation and grazing is trampling of the vegetation, which is accompanied by compaction of the soil.

The aim of this study is to determine the influence of the soil compactness on the development of the natural vegetation and on the distribution of some plant species. To obtain an impression of the influence of soil compactness, some indicative *Plantago* species were chosen for investigation, because soil compactness is thought to be of importance for the distribution of these species.

A synecological field study is expected to provide a basis for the formulation of a working hypothesis concerning the distribution of plant communities including *Plantago* species, and concomitant experimental work was started in 1971. Some aspects of the synecological study and the methods and preliminary results of the experimental studies are described here.

6.3.2. *Synecological study*

Some of the dune grassland vegetations of Voorne and Goeree are exposed to severe recreational pressure or grazing, mainly by cattle. Recreation takes place on the *Heveringen*, an old grassland formation in the dunes of Voorne, and grazing occurs, for instance, on the *Westduinen* on Goeree. The synecological situation is being studied mainly in those areas. This field work can be briefly described as follows.

The vegetation of sample plots is described annually by means of the Braun-Blanquet method. The plots were selected mainly along paths where *Plantago* species are present. The aim is to obtain an impression of the connection between the *Plantago* species and other plant species in relation to edaphic factors such as trampling and soil compactness.

Another aspect of this field work is the analysis of seasonal periodity and fluctuation of the species in the different localities in relation to different soil characteristics.

In the future the shoot/root ratio of the species under consideration will be determined. As an example, Table 11 shows the results of three vegetation analyses made in the *Westduinen* area, i.e. on a cattle path (plot I), just beside this path (plot II), and on a slight slope (plot III) close to these plots.

6.3.3. *Experimental work*

6.3.3.1. Material and Methods

To investigate the relationship between the distribution of plant species and various environmental conditions a distinction must be made between the germination and the development of the seedling. In this research

Table 11. Vegetation analysis performed in the Westduinen area of Goeree (1972)*.

I. Plot on a cattle path.
II. Plot just beside this path.
III. Plot on a slight slope close to the plots I en II.

Plot nr.	I	II	III
Size of the plot in cm	30 × 150	50 × 150	50 × 150
Cover herb layer in %	60	100	100
Cover moss layer in %	5	10	30
Height herb layer in cm	3	7	20
Trampling (+ present; ± sometimes; − absent)	+	±	−
Plantago lanceolata	r	+	2
Plantago major	3	1	
Plantago coronopus	r	1	
Potentilla anserina	+	+	+
Bellis perennis	2	2	2
Trifolium micranthum	+	1	1
Cerastium arvense	+	+	+
Carex panicea	1	3	2
Carex flacca	1	+	+
Agrostis tenuis	2	1	2
Anthoxanthum odoratum	+	2	2
Trifolium repens	2	1	
Ranunculus acris	+	+	
Spergularia rubra	+	+	
Linum catharticum	r	+	
Radiola linoides		r	r
Potentilla erecta		+	+
Festuca rubra		1	2
Briza media		1	+
Sieglingia decumbens		2	+
Luzula campestris		+	+
Rhytidiadelphus squarrosus		1	2
Brachythecium albicans		+	2

Species not included here:

I. *Festuca ovina* +, *Poa pratensis* +.

II. *Plantago intermedia* r, *Hierachium pilosella* +, *Hydrocotyle vulgaris* 2, *Hypochaeris radicata* +, *Lotus corniculatus* +, *Leontodon nudicaulis* +, *Cerastium holosteoides* +, *Crepis biennis* 1, *Viola rupestris* +, *Carex nigra* +, *Carex trinervis* 1, *Agrostis stolonifera* +.

III. *Lotus corniculatus* 2, *Euphrasia officinalis* 2, *Polygala vulgaris* 1, *Rumex* +, *Hypochaeris radicata* 2, *Ornithopus perpusillus* +, *Ranunculus bulbosus* +, *Viola rupestris* +, *Crepis capillaris* r, *Taraxacum erythrospermum* +, *Cynosurus cristatus* +, *Carex nigra* +, *Agrostis stolonifera* +, *Carex arenaria* +, *Cladonia rangiformis* +.

* Symbols: r: 1–5 specimens.
+: 5–100 specimens; basal covering 1–5 %.
1: many specimens; basal covering 1–5%.
2: basal covering 5–25%.
3: basal covering 25–50%.

period the influence of soil compactness was studied mainly on the basis of the germination of seeds of *Plantago major*, *Plantago lanceolata*, and *Plantago coronopus*, all collected on Voorne and Goeree in 1971, as well as the germination of seeds of *Plantago media* collected in Zuid-Limburg in 1972.

The seeds were stored in envelopes at room temperature. Germination occurred in plastic boxes ($37 \times 29 \times 10$ cm) filled with sand originating from the older dunes (humus content about 0.5%; pH ± 9). The tests were carried out in the greenhouse of the Biological Station. The substrate was chosen because all of the mentioned *Plantago* species except *media* are normally present on the older dune sand. *Plantago media* was included to permit comparison of the *Plantago* species occurring in the dunes with a plant species originating from another area.

The following treatments were applied. Three series were prepared: in series A the soil was not compacted, in series C there was a maximal compaction, and in series B moderate compaction. Compaction of the soil was achieved by ramming down the substrate. Soil compactness was determined by measurement of the soil resistance with a penetrometer. The soil resistance in the upper 2 cm was 0 kg/cm^2 in series A, 5 kg/cm^2 in series B, and 10 kg/cm^2 in series C. The percentage of water in the substrate was kept constant during the experiments. In each box two plant species were sown (200 seeds of each species).

For the controls, the seeds were placed on wet filter paper in petri dishes which were held in the dark in the greenhouse. The substrate and the filter papers were kept moist with distilled water. In the greenhouse the day temperature was about 27° C and the night temperature about 22° C.

6.3.3.2. *Results*

The results of these germination experiments are shown in Fig. 3 (mean values of two experiments). In total, the germination of 400 seeds per plant species was studied. The germination percentage showed no variation during the whole of 1972, except for *Plantago major*. The graph of *P. major* shows the results of two experiments, one carried out in August and the other in October, both performed in triplicate.

6.3.3.3. *Discussion*

The maximal germination percentage and the germination rate of the *Plantago* seeds differ between the substrates with different compactness. In general, the seeds on the dune soils without compaction showed the highest germination percentage and maximal germination was reached sooner than in the other series.

The lowest germination was generally obtained in the dune soils with the highest penetrometer resistance. For *Plantago major*, the difference between the maximum germination in series A (no compaction) and

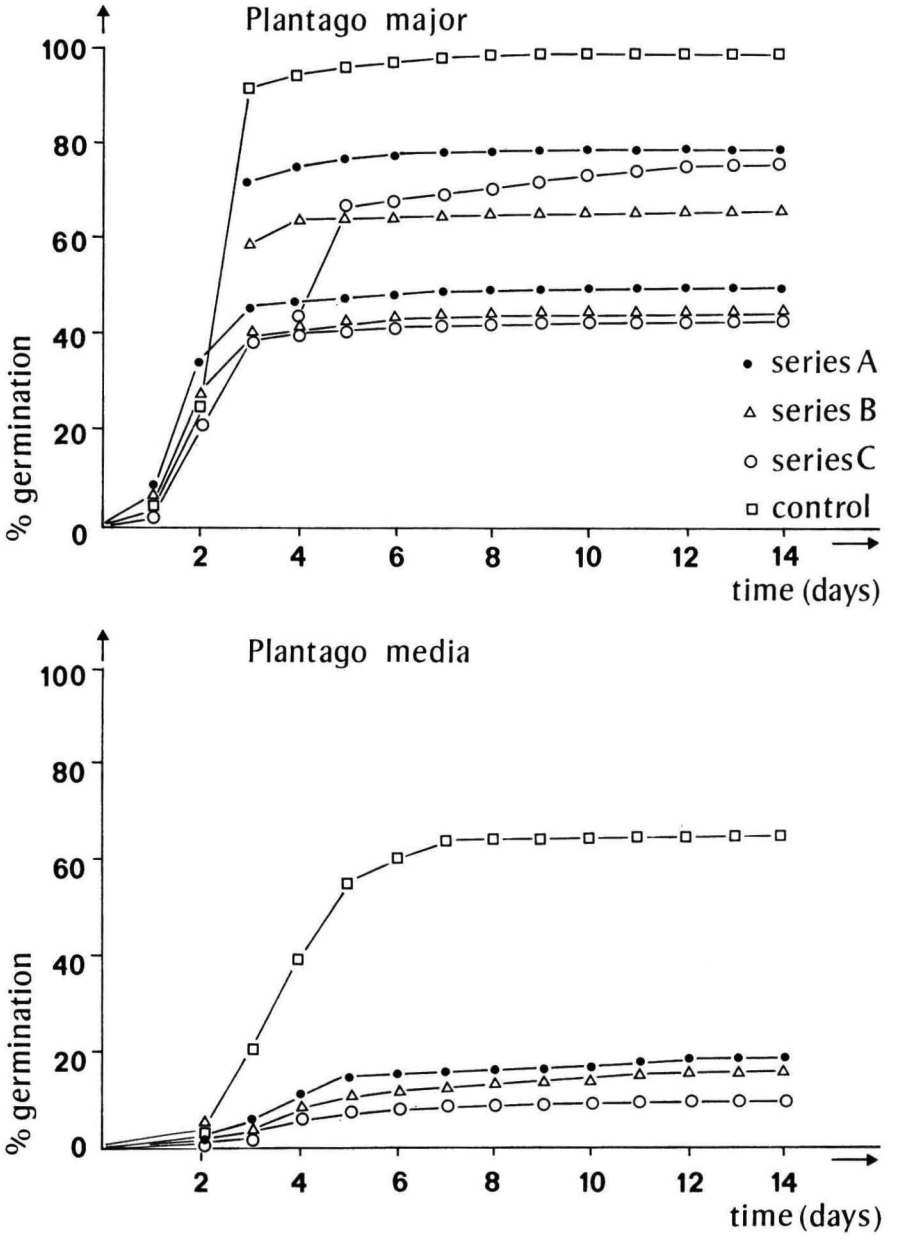
Plantago major
100
80
60
40
20
0
% germination
2
4
6
8
10
12
14
time (days)
• series A
△ series B
○ series C
□ control
Plantago media
100
80
60
40
20
0
% germination
2
4
6
8
10
12
14
time (days)

Fig. 3.

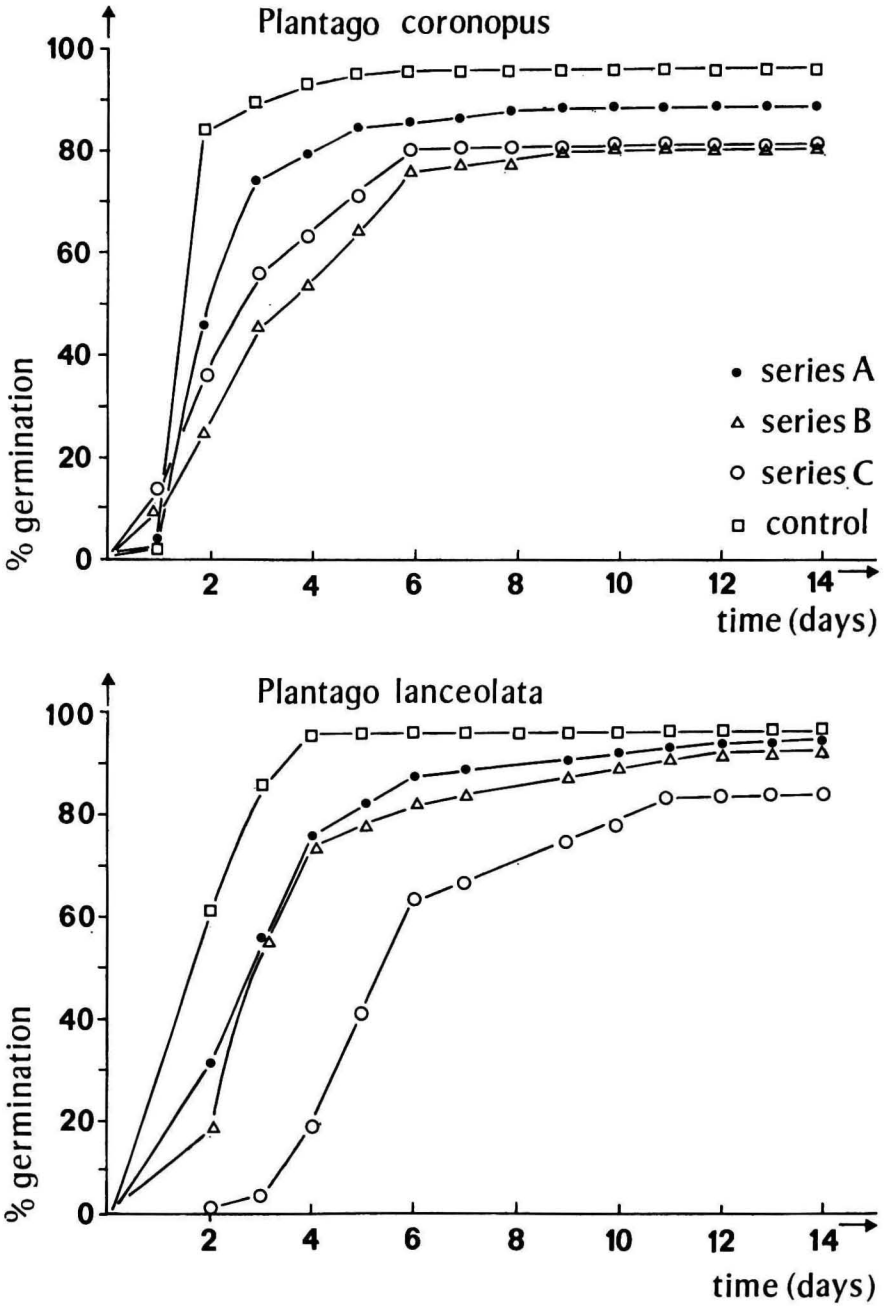

Fig. 3. Influence of soil compactness on the germination of four *Plantago* species.
Series A: Soil resistance 0 kg/cm².
Series B: Soil resistance 5 kg/cm².
Series C: Soil resistance 10 kg/cm².

series B (soil resistance 5 kg/cm^2) was significant, and similar differences were found for *Plantago media* between series A and C and B and C (X^2-test by Fischer; $P \leqslant 0.05$).

Germination of the seeds of *Plantago major* and *Plantago media* proved to be season dependent: in the winter under laboratory conditions these species showed no germination. The experiments will be continued at other times of the year and under additional conditions, for instance varying soil compactness during germination, and the direct influence of trampling will also be investigated.

J. W. WOLDENDORP

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LIMNOLOGICAL INSTITUTE, NIEUWERSLUIS

PROGRESS REPORT 1972

History and function of the institute

The Limnological Institute in Nieuwersluis, a small village between Amsterdam and Utrecht, dates from 1957. Research is done on the biological and chemical hydrography of the fens and lakes near the institute and also in the large fen region in the north-eastern part of the Netherlands (Tjeukemeer). Special study is made of the ecological relations between the organisms and their milieu, with respect to its chemical composition which changes both in depth and in time; also the relations between the various organisms are investigated. The institute organizes courses in limnology for students of the neighbouring universities in Amsterdam, Leyden and Utrecht. It takes an active part in the International Biological Programme (IBP); it organized an IBP panel meeting in 1966 and offers facilities to an IBP-Unesco fellow from abroad wishing to train or do research in limnology.

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INTRODUCTION

The studies on the lakes Vechten and Tjeukemeer were continued. The other projects included the food budget of *Daphnia magna* and the work on IJselmeer which was extended to the lake Ketelmeer.

Project-group Tjeukemeer

CHEMISTRY AND PRIMARY PRODUCTION: (Drs. H. de Haan, Dr. J. R. Moed, Dr. H. L. Golterman and Mr. R. F. Liqui-Lung)

At the end of the year 1971 the water of Tjeukemeer had still a rather high Cl^- concentration (4.65 meq.l^{-1}), indicating that – due to an unusually dry winter – the lake water had not been replaced by polderwater. The Cl^- concentration decreased steadily to 2.6 meq.l^{-1} in April, but before the Tjeukemeer contained polderwater only, the IJselmeerwater was let in again leading to a Cl^- concentration of 5.4 meq.l^{-1} in October 1972. The IJselmeerwater contained 11 meq.l^{-1} of Cl^- on 17 April, which gradually decreased to 6.4 meq.l^{-1} in August. The hydrological regime in 1972 is reflected in the abnormal fluctuation in the concentration of most of the nutrients. The temperature of the lake showed an almost

linear increase from 2° C in March to 19° C on 7 August; afterwards it decreased linearly again to 4° C in December. Although differences with previous years are small, temperatures were lower this year. As a result of the hydrological situation the NH_3–N concentration increased in January from 400 $\mu g.l^{-1}$ to 1100 $\mu g.l^{-1}$ on 23 February, after which it sharply decreased to a value of 25 $\mu g.l^{-1}$ in March. NO_3–N was stable at 1 $mg.l^{-1}$ in January, increased only very little in early March and decreased afterwards to 50 $\mu g.l^{-1}$ (17 April). In November both NH_3 and NO_3 increased: NH_3–N to 1–1.5 $mg.l^{-1}$ and NO_3–N to 600 $\mu g.l^{-1}$. NH_3–N fluctuated between 200 and 300 $\mu g.l^{-1}$ in summer.

The dissolved phosphate (Tot–$P_{diss.}$) never fell below 20 $\mu g.l^{-1}$. It was low in January/February due to comparatively little amount of polderwater let in. It increased to the usual average values of 150–200 $\mu g.l^{-1}$ in December. PO_4–P remained rather low during the whole summer with values mostly around 5 $\mu g.l^{-1}$. The big differences between Tot–$P_{diss.}$ and PO_4–P may be explained by a poor availability for plankton of Org–$P_{diss.}$ as compared with PO_4–P or by considerable release of organic phosphates by decaying phytoplankton. The concentration of particulate phosphate (Part.–P) is mostly about 100 $\mu g.l^{-1}$, with sharp peaks on 5 April and on 29 May (200–300 $\mu g.l^{-1}$). Part–P is partly cellular and partly detrital (including humic) phosphate.

The silicate (SiO_2–Si) concentration in January was low, due to the small amount of polderwater in the lake. It decreased gradually up to 5 April to 100 $\mu g.l^{-1}$. Sudden sharp peaks (around 1.0 $mg.l^{-1}$) in July and August cannot be explained. No diatom growth took place in this period. Summer values were in general not low. A high concentration was found in IJselmeer, but before this water reached Tjeukemeer, the silicate is removed in the Groote Brekken. In the surrounding polders high concentrations were found, especially if there is no water flow.

Chlorophyll increased from 40 $mg.m^{-3}$ in winter to 120 $mg.m^{-3}$ in summer. Assuming chlorophyll to be 1½% of the dry weight, this increase corresponds with an increase in dry weight of 5.6 $g.m^{-3}$ and with 0.6 $g.m^{-3}$ of Particulate–N. As the decrease in the sum of ($NH_3 + NO_3^-$) is 2 $mg.l^{-1}$ this year shows again a nitrogen deficit for which no explanation other than denitrification can be given. The chlorophyll concentration showed two peaks one in early April and the other in end of May. On both occasions the primary production was relatively low and the respiration high. The primary production maximum coincided with the chlorophyll minimum.

Mean gross primary production was 5.7 $g.m^{-2}.d^{-1}$ of O_2 during the vegetative period, well in the range of values for 1971 and 1972.

The net primary production showed a negative value for the first time. Unfortunately this year no data is available on the magnitude of the fraction of the algal oxygen uptake compared to the total value. If the value of the O_2 uptake can be considered to be 50% of the gross primary production, as seen in the values for 1970 and 1971, we come to a calcu-

Table 1. Summary of primary production data for the years 1970–1972. Note the differences in the net production.

	Gross primary production	O_2 uptake	Net primary production
1970	8	3.5	5.5–6.3 [2]
1971	5	2.4	2.6
1972	5.7	7.3 [1]	−1.6

[1] "DCMU" instead of "dark" bottles (Golterman, 1971).
[2] Calculated with an algal O_2 uptake measured to be 50% of the total O_2 uptake.

lated value of O_2 uptake in 1972 of 2.8 g.m.$^{-2}$d^{-1}, resulting in a net primary production of 2.0 g.m.$^{-2}$d^{-1}. This lower value may be explained by the different technique used, but is probably due more to different hydrological regime this year, leading to a higher concentration of readily oxidizable organic matter. The COD values of the particulate matter expressed as C are 100% higher than those calculated from chlorophyll (chlorophyll × 35 = mg C) both on 5 April and 29 May. Apparently there is a great need for determining cellular and detrital carbon separately.

ALGOLOGICAL STUDIES (Dr. J. R. Moed)

Moed studied the periodicity of Si-requiring algae of Tjeukemeer and of a small pond, Poepegat, close to this lake.

In Tjeukemeer, *Melosira* spp., *Cyclotella* sp., *Stephanodiscus* sp. and *Diatoma elongatum* formed the main diatom flora in early 1972. The population of *Diatoma* disappeared about 5–6 weeks later (maximum cell density 18.000 cells per ml on 25 April) than the other ones (maxima on 15 March). The well recognizable silica structures of the cell wall of *Diatoma* may be indicative of its physiological state. This evoked Moed's interest to study its population dynamics. By performing chemical analysis of lake water and by replenishing the nutrients which are likely to be depleted an attempt was made to get information concerning factors leading to lethal effects. During the period of dying of *Diatoma*, microscopic observations showed a changed appearance of its chromatophores.

In Poepegat populations of the Si-requiring Chrysophyceae *Synura* sp. and of the diatoms *Cyclotella* sp. and *Diatoma elongatum* var. *tenuis* were counted. In this case too, the numbers of *Diatoma* decreased later (one week) than those of the other algae mentioned.

Further, for simulation experiments a medium was developed which, although ignoring the humus content, was very similar to the relevant Tjeukemeer water. In addition, a number of clones of *Diatoma* originating from Tjeukemeer, were isolated. It appeared that the size of the cells of *most* of these clones diminished, in an irregular way.

For the other algae in Tjeukemeer, the same species – but for the blue green algae – showed blooms as in the previous years. *Scenedesmus* showed one peak (28,000 cells per ml) on 29 May, but was persistent in the lake throughout the year varying between 2,000 and 6,000 cells per ml. *Pediastrum* recorded between 100 and 200 cells per ml during the year with a peak of 1300 cells per ml on 30 May.

Threads of the blue-green alga *Oscillatoria* reached a maximum of 80.000 cells per ml on 15 May, decreasing till the end of June to 10.000 threads per ml and showing a second peak in October/November. *Oscillatoria redekei* and *O. limnetica* contributed equally to the spring maximum of *Oscillatoria* spp., while the former named species constituted about 50% of autumn maximum of blue-greens the other half being formed by *O. agardhii* and *Aphanizomenon flos-aquae*. *Microcystis aeruginosa* was very sparsely recorded.

HUMIC ACIDS: (Drs. H. de Haan)

De Haan proposed a method for the determination of soluble humic compounds in fresh water based on its tyrosine content. It seemed that the amount of HC as Pt-units is not directly proportional to this tyrosine content. The occurrence of relatively large humic compounds molecules is reflected in a large ratio of tyrosine-units over Pt-units, since this ratio is positively correlated with the ratio between the optical density at 250 and 365 nm in fresh water. Though the method together with colour measurements as Pt-units gives information about the quantity and nature of humic compounds, it is not suitable for analysis of humic compounds in fresh water.

Once a month the ninhydrine nitrogen, the orcinol hexoses and the amount of phenolic groups have been determined in the three main fractions obtained from a Sephadex G 25 gel filtration of humic compounds from Tjeukemeer. The results per colour unit show a dynamic nature. The fraction with the largest particle size shows a more stable behaviour in this respect than the two fractions with the smaller particle size. Low hexose content of all three fractions could be correlated with high chlorophyll-*a* contents in the lake. Ninhydrine nitrogen and phenolic groups maxima could be correlated with chlorophyll *a*. The results indicate that humic compounds in fresh water act as a regulating factor in the turnover of nutrients, but it should be realized that polder water is the main source both of humic acids and nutrients.

According to experimental data, two different microbial processes in which coloured compounds are involved may occur in Tjeukemeer. One process is characterized by colour increase and the other by colour decrease during incubation with a bacteria isolated on humic acids. The latter process is stimulated by lactate and indicates the existence of microbial co-metabolism of humic compounds in Tjeukemeer. Organic matter produced by algal growth and the humic compounds themselves are supposed

to be of importance in determining which of the two processes dominates in the lake.

The influence of the fraction with the largest particle size on the growth of a *Pseudomonas* sp. from Tjeukemeer was studied. The addition of this fraction to a mineral medium, in which lactate was the only organic C-source, increased the cell yield of the *Pseudomonas* by 30%. The cell yield per Mol of metabolized lactate increased by 40%. The *Pseudomonas* does not grow on a medium in which the fraction studied was the only organic C-source. Furthermore, the chemical changes in the fraction could be established during the growth of the *Pseudomonas* in a lactate medium with humus added. These two effects may explain the increased cell yield in terms of co-metabolism of the added humic compound fraction by the *Pseudomonas* sp.

ZOOPLANKTON: (Drs. J. Vijverberg and Dr. R. D. Gulati)

Vijverberg continued his study of zooplankton production in Tjeukemeer by means of population dynamics method.

Absolute population densities with variation in space and time and population structure and number of eggs per female were assessed.

Fourteen species of Copepoda and Cladocera all of which are of numerical importance in the Tjeukemeer were cultured at at least three different temperatures. In all six different temperatures were used (2.5, 5, 10, 15, 20 and 25° C). Using data from these experiments, growth, birthrate and longevity were calculated.

Biomass was determined by chemical methods and was expressed as μg per individual for different size groups of each species (or taxonomical group). The following substances were determined: protein, fat, sugars, Kjeldahl-nitrogen and phosphate. The C.O.D. was also determined as an estimate of total organic carbon.

In an experimental culture the foodlevel proved to be sub-optimal for the growth, egg production, maturity and longevity of *Daphnia hyalina*; this seemed to be the result both of a poor food quality and a sub-optimal algal biomass in the lake. The factors such as growth, number of egg per female, number of newborn per female per week, size at maturity and longevity were all influenced by foodlevel. The difference in numbers of newborn per female per week could not be explained by the difference in clutch size only, and it was shown that, when the foodlevel was low, this was also influenced by the length of the eggless period between two batches.

Furthermore, Vijverberg summarized 4 years data (1968–1972) on zooplankton densities:

1) Five species of Copepoda and eight of Cladocera are abundant and account for 98% of the total numbers.

2) Cladocera account for 75% of the total numbers except in early spring when Copepoda are dominant.

3) Variation in abundance during the successive years is small. However, in 1969 large aberrations were found due probably to sand digging and to concomitant increase in turbidity.

4) The occurrence *) of the species was as follows:

Very abundant:	*Bosmina coregoni* (May–November)
	Chydorus sphaericus (May–January)
Abundant:	*Acanthocyclops robustus* (March–January)
	Bosmina longirostris
	Daphnia hyalina (May–October)
Moderate:	*Mesocyclops leuckarti* (March–November)
	Eurytemora affinis
	Cyclops strenuus/vicinus
	Daphnia cucullata
	Ceriodaphnia pulchella
Rare:	*Diacyclops bicuspidatus*
	Diaphanosoma brachyurum
	Leptodora kindtii

Besides this study on production, some field work was done on thirteen major Frisian lakes and two small waterbodies. The species composition in the different Frisian lakes was very similar. However, Cladoceran densities differed among these lakes. The densities in the Slootermeer, the Brandemeer and De Leyen were high, whereas low densities existed in Tjeukemeer, Heegermeer, Groote Brekken and Koevordermeer. There was a positive correlation between chlorophyll-*a* content and cladoceran density.

Vijverberg expects that the experimental results on the Copepoda will become available by the end of 1973, and for the Cladocera during 1974. His field data will be ready for publication in 1975 and his data on population dynamics and production in 1976.

Role of filter feeding zooplankton as primary consumers of phytoplankton in Tjeukemeer

Gulati started work on the production of the entire community of filter feeding zooplankton, their food consumption, assimilation and their respiration rates in March 1972. Lake phytoplankton was used as tracer food. The experiments were performed in the laboratory under conditions similar to those in the field.

The data (see table 2) over the period March/February 1972/1973 indicate that the standing crop of zooplankton rose from 1.5 g.m^{-2} in

*) If no period is given, occurrence is only for a few months.

spring to over 4 $g.m^{-2}$ in summer and thereafter continuously decreased through autumn to 0.5 $g.m^{-2}$ in winter. *Chydorus sphaericus*, *Bosmina coregoni*, *B. longirostris* and *Daphnia hyalina* were the important forms contributing practically to the whole production in summer.

Table 2. (1972–1973 (upto end February))
Mean phyto- and zooplankton standing crop (D. weight) of Tjeukemeer and daily food budget of zooplankton. Mean lake depth is 2 metres.

Parameter	Spring	Summer	Autumn	Winter	Mean
Phytoplankton ($g.m^{-2}$.)	24.95	33.8	33.4	20.93	29.7
Zooplankton ($g.m^{-2}$.)	1.55	4.4	0.90	0.66	2.5
Consumption of phytoplankton by zooplankton (d^{-1}, %)	2.1	5.6	0.60	0.30	2.94
Consumption ($g.m^{-2}.d^{-1}$)	0.50	1.82	0.23	0.06	0.93
Assimilation ($g.m^{-2}.d^{-1}$)	0.20	0.70	0.09	0.01	0.36
Respiration ($g.m^{-2}.d^{-1}$)	0.14	0.53	0.05	0.01	0.27
Production ($g.m^{-2}.d^{-1}$)	0.06	0.17	0.04	0.00	0.09
Daily ration (%)	32	41	25	9.5	30
K_1 (%)	12	9.3	17.4	0	9.4
K_2 (%)	30	24.3	44.4	0	24.3
Transfer efficiency (%) *	14	31	33	0	22

* Net primary production / net production of primary consumers × 100.

Phytoplankton: zooplankton (biomass) ratios in the Tjeukemeer vary from 16 : 1 in spring to 8 : 1 in summer and to 30 : 1 in autumn and winter. The zooplankton average of 2.5 $g.m^{-2}$ for the 25 fortnightly estimates during the year 1972/1973 was approximately 8% of the phytoplankton biomass (30 $g.m^{-2}$) during the same period. Nearly 3% (0.9 $g.m^{-2}.d^{-1}$) of the phytoplankton standing crop is daily consumed by zooplankton, i.e. 2.1, 5.6, 0.6 and 0.3% respectively, for spring, summer, autumn and winter.

Gross zooplankton production (0.36 $g.m^{-2}.d^{-1}$) was about 40% of the consumed food; nearly 75% of this production is utilized for metabolic needs (respiration) leaving 0.09 $g.m^{-2}.d^{-1}$ for net daily increment. Nearly 82% of the total zooplankton production (32 $g.m^{-2}$) took place in summer (26 $g.m^{-2}$). P/B ratios were 2.0, 6.0, 2.5 and 0.0 during spring, summer, and winter, respectively. Transfer efficiency, as the part of net primary production transformed into secondary production was 22%.

Phyto- and zooplankton comprized 43% of the total organic C in the lake but 60% of total organic N in the lake. This could be attributed to the dominance of blue-greens and thus higher N content fixed in algae. The origin of high dissolved C in the lake is in detritus and allochthonous materials. Both phyto- and zooplankton had nearly the same N as % of plankton bound C, i.e. 13–14% in both cases.

BENTHIC ANIMALS (D. M. Beattie, M. Sc.)

Beattie summarized the field work of previous years and established that a common feature of the larger Friesian lakes is their low densities of chironomids, this being particularly so for Tjeukemeer. This low density in Tjeukemeer is caused not by overpredation by fish, but rather by the physical characteristics of the lake – its shallowness and exposure to wind.

The fish which is normally expected to predate on chironomids is now forced to depend on an insubstantial diet of zooplankton. Furthermore, it has emerged from studies that the diapause in several species of different genera is an important factor controlling the life cycle. This problem will be approached experimentally in the laboratory.

Project-group Lake Vechten

CHEMISTRY AND PRIMARY PRODUCTION: (Dr. H. L. Golterman, Mr. R. F. Liqui-Lung and Drs. H. Verdouw)

Routine chemical analyses were carried out, except for the determination of nitrogen compounds, which was interrupted again due to the illness of the technical assistant concerned. The investigation of the hypolimnion by taking mixed samples gave insufficient information. More data is needed on the actual gradient of compounds which show strongly changing concentrations in the hypolimnion.

Work on the sediment-water exchange of the different cations was taken up again by Verdouw who developed a method to obtain samples from the interstitial water at different depths in the sediments under anaerobic conditions. Adsorbed cations on the sediments could be estimated after extraction with 2 M KCl, and adsorption characteristics of Ca^{++}, Mg^{++}, Fe^{++}, Mn^{++} and NH_4^+ thus be calculated. As the present method for measuring ammonia might be disturbed by the presence of labile organic nitrogen-compounds, Verdouw worked out a specific enzymatic determination based on the following reaction:

$$\alpha\text{-Ketoglutaric acid} + NH_4^+ + NADH \xrightarrow[\text{dehydrogenase}]{\text{glutamate}} \text{glutamate} + NAD^+ + H_2O.$$

During summer stratification both the start (May) and maximal development of the anaerobic condition were earlier than in the previous years. This might be caused by the greater rate of warming up this year. No sulphate could be detected in the hypolimnion from July to October. H_2S was detectable a few times. Also, Fe and Mn were present in solution earlier than normally, but the maximum values reached were not different from those of previous years.

Liqui-Lung measured between March and October the primary production which ranged from 97 to 645 with a mean value of 350 $mg.m^{-2}.d^{-1}$ of C. Between 24 May and 30 August the photosynthesis-depth curve showed maxima at two depths due probably to an inhomogeneous distribution of phytoplankton. The efficiency of photosynthesis ranged from 0.06 to 0.21% of the total incident radiation.

The efficiency was negatively correlated with the light intensity up to 1600 $Kcal.m^{-2}$ per 4 hr but above this value the correlation was positive. In the latter case the photosynthesis in the water column between 3 and 7 m contributed more to production than that in the 0–3 m layer did.

On 16 August a 0.7 m thick layer of *Oscillatoria* sp. was observed below the thermocline for the first time. The cells performed intensive photosynthesis when exposed to surface light conditions. The light attenuation coefficient ε varied between 0.4 and 0.6, so that light values at 3 and 7 m were respectively 200–700 and 20 $Kcal.m^{-2}.4\ hr^{-1}$.

Photo-inhibition was observed on nearly all sampling days; A_{max} occurred mostly between 1 and 2 m and the production range from 16 to 88 $mg.m^{-3}.4\ hr^{-1}$ of carbon (mean value 37).

A linear correlation existed between A_{max} and Tot–P (correlation coefficient 0.96).

ZOOPLANKTON (Dr. R. D. Gulati)

The secondary productivity study on lake Vechten was carried out simultaneous with that on Tjeukemeer, using similar techniques.

Zooplankton had a mean content of 2 $g.m^{-2}$ and comprized 25% of the biomass of phytoplankton (7.6 $g.m^{-2}$). Daily consumption of phytoplankton by zooplankton was 5%. Zooplankton average biomass was the highest in spring (2.9 $g.m^{-2}$) resulting in spring consumption rate by zooplankton of 0.75 $g.m^{-2}.d^{-1}$ and net zooplankton production of 0.13 $g.m^{-2}.d^{-1}$. Summer averages of consumption, 0.48 $g.m^{-2}.d^{-1}$, and production, 0.12 $g.m^{-2}.d^{-1}$, were similar to the respective means of the entire study period (March–November).

A total production of 18 $g.m^{-2}$ in 56 days during spring was nearly the same (19 $g.m^{-2}$) as that in 156 days in summer. About 22% of net primary production was transferred from phytoplankton to net secondary production of filterfeeding zooplankton.

Zooplankton standing crop as organic carbon (1 $g.m^{-2}$, study period average), constituted 25% of the algal standing crop as organic carbon; respective figure for organic nitrogen in zooplankton was much higher, *viz.* 43% of the nitrogen in phytoplankton. About 80% both of the total organic C and N in the lake was present in the dissolved form and the rest as phyto- and zooplankton C and N. However, particulate N as % of total organic N was much higher in spring and autumn, respectively 38 and 26% of total organic N. This could be correlated to higher inorganic

N as NH_3 and as NO_3 values in the water both during spring circulation and during autumnal breakdown of thermal stratification.

MICROBIOLOGY: (Drs. Th. E. Cappenberg)

As reported in the previous years Cappenberg directed his studies to an understanding of ecological and physiological relationships between sulphate-reducing and methane-producing bacteria in the mud-water interface.

It is concluded from distribution-studies in mud cores that sulphate reducers are most abundant at a depth of 0 to 2 cm at pS^{-2} values of about 11 and redox potential of -100 to -150 mV. Maximal numbers of methane producers occurred at a depth between 3 and 6 cm in the mud, where the pS^{-2} values was 14, redox potential -250 to -300 mV, and the concentration of methane was maximal. The concentration of sulphate-ions in the sediments are the limiting factor for the sulphate reducers. When these bacteria have used up the sulphate-ions, the methanogenic bacteria develop in greater numbers.

Cappenberg did experiments on selective inhibition as well as enhancement of methanogenesis with relevant substrates. This coupled with selective countings of the methane producers showed that acetate-fermenting types comprise 75% of the mixed populations of the methanogenic bacteria. These bacteria are found at about 5 cm in the mud where pS^{-2} value (13 to 14) was similar to that found in cultures – a value found to be optimal for the methanogenic bacteria.

The concentration of acetate increased if the mud was incubated with CCl_4 or with $CHCl_3$, as does that of lactate when incubated with β-fluoro-lactate. These findings lead to assumption that lactate may be used as an energy source by *Desulfovibrio*, provided both the inhibitors are selective. The biomass of *Desulfovibrio* increased in chemostat experiments with lactate as limiting C-source when methane was added, suggesting that methane is metabolized by *Desulfovibrio*.

BENTHIC ALGAE: (Drs. C. L. M. Steenbergen)

Steenbergen studied the benthic algae of lake Vechten and made a list of epiphytic diatoms from three sampling stations. Further, he spent most of his time in writing up the results of his work on *Scenedesmus* in synchronized culture.

Other Projects

1) IJSELMEER: (Drs. W. de Kloet)

De Kloet carried out 4 trips on the Ketelmeer in order to study the accumulation of phosphate during the period October 1971 to March 1972. The total phosphate-concentration in the IJssel was on the average 300 $\mu g.l^-$ higher than in the previous summer. There was a loss of 27% in

the phosphate concentration during the passage of water through the Ketelmeer into the IJselmeer. During calm periods there was a decrease of up to 30%, and in the summer a decrease of 42–47% was found. In IJselmeer (at station VII) the concentration was 75% lower. The same pattern was found for inorganic phosphate. The concentration of PO_4–P in IJselmeer was on the average 75% lower than in Ketelmeer. In Ketelmeer the decrease of PO_4–P was much less in winter than in the summer with a mean value of 22% (summer 40%). This difference may partly be due to the absence of phytoplankton in Ketelmeer during winter. A linear correlation between dissolved Fe and PO_4–P concentration as well as between total Fe and particulate phosphate was found. The concentration of Part–P showed a decrease, possibly due to sedimentation, from IJssel to Ketelmeer and from Ketelmeer to IJselmeer. However, in the Ketelmeer itself this phosphate concentration remained constant.

In summer the primary production was on the average in the Ketelmeer (Ketelhaven-IJssel) 0.5 $g.m^{-2}.d^{-1}$ of C and in the IJselmeer 2–3 $g.m^{-2}.d^{-1}$ of C. In Ketelmeer a gradual increase in the direction of IJselmeer was found. On almost all the occasions it appeared that in the entire study area 80% of the production took place in the upper 1 m layer. In Ketelmeer the production decreased to 0.02 $g.m^2.d^{-1}$ of C in November, and in IJselmeer a decrease to 0.2–0.3 $g.m^2.d^{-1}$ of C was noted a month earlier. The fall in production rates in both cases is simultaneous with lowered production efficiences. Furthermore, a correlation between primary production and chlorophyll was recorded both for Ketelmeer and for IJselmeer.

2) ZOOPLANKTON: (Dr. R. D. Gulati)

Gulati investigated the food budget of *Daphnia magna* using C^{14} labelled *Chlorella vulgaris* as food. The efficiency indices thus calculated indicate that this cladoceran consumes 30 to 50 μg of organic matter mg^{-1}.D.W. hr^{-1}. About 60% of the consumed food, i.e., 18–30 $\mu g.mg^{-1}.D.W.hr^{-1}$, is absorbed in the digestive tract and the remainder is egested as unutilized or partly digested material. A study of metabolism demonstrates that respiratory losses account for 7–10 $\mu g.mg^{-1}.D.W.hr^{-1}$; and the difference between total metabolic losses and respiratory losses is due to loss on ecdysis, i.e., periodic loss of excuvial and moulting fluids. These losses are approximately 2 $\mu g.mg^{-1}.D.W.hr^{-1}$. The rest 9–18 $\mu g.mg^{-1}.hr^{-1}$ is utilized as somatic and generative growth of the animal.

Irrespective of temperature, which was kept constant at 20° C, the specific growth, assimilation and specific consumption rate constants were two times higher in summer than in winter. Discrepancies between cumulative and instantaneous food budgets appear to be due mainly to experimental errors, e.g., in the estimation of respiratory losses and in converting the COD data on food (*Chlorella vulgaris*) and *Daphnia* to dryweight figures using constant conversion factors – irrespective of the physiological state of food and of the age of *Daphnia*.

A study of turnover rates of body carbon in *D. magna* revealed that about 6 to 7 $\mu g.mg^{-1}$ body carbon is turned over hourly in winter at 20° C. The summer turnover rates under similar condition (temp. 20° C) are 30 to 50% faster than in winter. The turnover rates in starving animals were twice as fast as in those fed during the experiments. Loss of carbon, N–NH_3 and P–PO_4 in starving animals showed adaptation with time through reduced respiratory and excretory rates.

An experiment run for four days revealed that carbon losses which during the first two days (t_0–t_2) averaged 2.89 $\mu g.mg^{-1}.D.W.hr^{-1}$ were reduced to 1.10 $\mu g.mg^{-1}.D.W.hr^{-1}$ during the subsequent two days (t_2–t_4). Corresponding decrease in the excretory losses of NH_3–N and P–PO_4 were: from 0.43 to 0.28 $\mu g.mg^{-1}.D.W.hr^{-1}$ and from 0.07 to 0.04 $\mu g.mg^{-1}.D.W.hr^{-1}$, respectively during the periods t_0–t_2 and t_2–t_4; in other words conservation of carbon (probably through reduced respiratory rates) appeared to be most significant, though P and N also decreased appreciably in the metabolic wastes.

Prefeeding on *Microcystis* homogenate and on its "toxin" extract showed that the so -called toxin may effect the feeding of *Daphnia* in two ways: first, by momentarily inactivating the animal's filtration and feeding processes, so that on return to normal food condition animals feed much faster; secondly, in higher dozes the toxin results in increased metabolic disturbance and mortality, perhaps due to a partial damage to the feeding mechanism of the surviving population.

Continuous feeding of *Daphnia* in the same culture medium for some days has in some way an inhibitory effect on their feeding mechanism. Refreshing the culture medium has a stimulating effect on feeding resulting in an increase in the feeding rates both in prefed and prestarved animals. Maximum increase measured was 9 times.

There was a linear relationship between organic N and organic dryweight of *Daphnia*, N as a % of D.W. was 9% on the average.

Mr. F. van Zeland, under the guidance of Gulati, carried out a study on the nitrogen excretion in *Daphnia magna*. The excretion rates of adult animals with a biomass of 65.0 ± 16.8 μg and N content of 7.3% was: Total–N, $0.71 + 0.12$ $\mu g.mg^{-1}.b.w.hr^{-1}$; and NH_3–N, $0.45 + 0.12$ $\mu g.mg^{-1}.b.w.hr^{-1}$. The excretion of NH_3–N constituted about 62% of total nitrogen loss. The rise of temperature from 20 to 30° C significantly affected both the ammonia and total N excretion.

Starving animals did not reveal any specific adaptation to cut down the excretory losses in nitrogen. Before a large scale mortality occurred in the starving animals both N content and biomass of the animals was 41% of the starting figures.

H. L. GOLTERMAN
R. SOEKARJO

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DELTA INSTITUTE FOR HYDROBIOLOGICAL RESEARCH YERSEKE

PROGRESS REPORT 1972 *

In 1957 the Division of Natural Sciences, reacting on an initiative of the Commission for Ecology, created an institute, to be established in the deltaic area of the south-west Netherlands, with the aim of studying the biological changes to be expected as results of the closing of the various river-mouths and sea-arms in this area.

When the Zuiderzee was closed by a dam and converted into the fresh Ysselmeer between 1920 and 1950, extensive biological research was carried out by a group of fishery biologists, members of botanical and zoological societies and academic staff, under the direction of dr. H. C. Redeke. The important results obtained during this study, warranted the expectation that in the more diversified deltaic area of the rivers Rhine, Meuse and Scheldt, even more results could be achieved, especially so when one agency, located in the area, was given the task to make a co-ordinated effort to study the problems from various angles. After an exploratory phase, in which qualitative distribution of biota is to be studied from an ecological point of view, experimental work will be initiated, in order to elucidate the causal background of the changes observed.

The institute was erected under the name "Division Delta-Research of the Hydrobiological Institute", with the object to stress its affiliation to the Hydrobiological Institute at Nieuwersluis. As both institutes grew and matured the difference between the studies carried out in both of them gradually became apparent and in 1968 it was decided to change both names into their present form.

The institute is located at Yerseke on the Oosterschelde, the sea-arm to be closed in the last stage of the s.c. "Delta plan".

The exploitation of the institute is financed by means of funds allotted to the Academy.

Scientific Staff

Dr. K. F. Vaas – Director
F. Vegter – Chemist
Dr. W. G. Beeftink – Botanist (Phanerogams)
P. H. Nienhuis – Algologist
R. Peelen – Planktonologist (Northern Delta area)
C. Bakker – Planktonologist (Southern Delta area)
Dr. S. Parma – Experimental Planktonologist
Mrs. C. H. Borghouts – Zoologist
W. J. Wolff – Zoologist
Dr. A. G. Vlasblom – Experimental Zoologist
A. B. J. Sepers – Microbiologist

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* Communication nr. 110 of the Delta Instituut voor Hydrobiologisch Onderzoek, Yerseke, The Netherlands.

I. Introduction

The present year was our first experience with our new surroundings. Various minor deficiencies came to the fore, mainly concerning installations for the constant temperature rooms, for demineralized water etc. Repair and control required sometimes a good deal of effort on the part of the technical staff.

However, we also fully enjoyed the better accommodations offered by the new and modernized apartments.

This year's progress in the technical works carried out by the Department of Roads and Waterways, and, owing to the changes involved, having consequences for the work of the institute, comprised additional constructions on the sluices in the Volkerakdam, the work on the canal connecting the rivers Scheldt and Rhine and the constructions in the mouth of the Oosterschelde. Here the new island Noordland was built and was connected with the artificial island on the sandflat Neeltje Jansplaat, by means of a dam.

II. Descriptive Research

1. CHANGES IN ENVIRONMENT AND BIOTA IN VARIOUS REGIONS

1.1. *Brielse Meer, Brielse Gat*

In the Brielse Meer the Department of Roads and Waterways started an experiment on technical scale by adding iron salts to the inflowing water. The result was a decrease in phosphates down to 10–20% of the original concentration.

Blue-green algae were less abundant in the plankton than in previous years. However, as the amount of insolation was extremely low this year and moreover the lake receives a quantity of unpurified sewage from the town of Brielle amounting to 10,000 population equivalents, a direct connection between the decrease in blue-greens and phosphates can not yet be established.

In the Brielse Gat, at a chlorinity fluctuating around 11‰, Mr. R. Peelen found the holotrichous ciliate *Cyclotrichium meunieri* for the first time. This protozoan is closely related to *Mesodinium pulex* which appeared in Lake Veere in 1965 at a similar salinity.

Since the closure in 1966 the higher vegetation changed considerably. Some halophytes, such as *Elytrigia pungens* are expanding on substrates containing clay, but contracting on sandy substrates. After six years a relatively steady state is not yet reached. Grazing by cows prevents the vegetation from becoming monotonous owing to the expansion of *Hippophae* and other shrubs.

1.2. *Haringvliet, Hollands Diep, Nieuwe Merwede, Biesbosch*

In the fresh basin behind the dam through the mouth of the Haringvliet, the concentrations of various inorganic nutrients proved to have

risen and the amount of oxygen, dissolved in the water, to have fallen. However, whether this change from the estuarine conditions of 1969 is a temporary or a permanent one, can not yet be decided as the river discharge was extremely low this year. The Rhine dropped to a value of 1/3, the Meuse even to 1/6 of their average discharges.

In the Biesbosch a well developed freshwater plankton could be observed, in the Hollands Diep the diatoms *Asterionella formosa*, *Melosira granulata* and *Stephanodiscus* spp., began to develop and reached large numbers further on in the Haringvliet. The blue-green algae *Microcystis aeruginosa*, *Aphanizomenon flos-aquae* and *Oscillatoria agardhii* were found for the first time in these waters in summer and autumn, *Oscillatoria* in small numbers only.

According to Mr. P. H. Nienhuis the littoral algal vegetation still remains poor and shows all characteristics of a young, unstable environment, dominated by green- and blue-green algae. The tidal alga *Blidingia minima* has maintained its position owing to the slight tidal movements in the area via Nieuwe Waterweg, Oude Maas, Spui and Dordtse Kil.

Halophytes such as *Plantago maritima* and *Puccinellia maritima* growing on meadows before the closure are nearly absent now, being replaced by *Festuca rubra*, *Trifolium repens*, and in a later stage, *Poa trivialis*. Stands of *Phragmites australis* and *Scirpus* spp. showed an impressive increase, but the latter suffered from wind and waves in some places. On the Slijkplaat, totally without vegetation in 1970, Dr. W. G. Beeftink counted 43 species of higher plants in 1971 and 78, including 19 planted species, in 1972. In view of the valuable possibilities of the island as a habitat for interesting plants and birds the decision of the Department of Crownlands to plant willows, poplars and ornamental shrubs is a regrettable one.

According to measurements carried out by Mr. M. C. Daane with soil-water tubes, the salinity of the soils of the Kwade Hoek, a salt marsh area outside the dam through the Haringvliet, has doubled because no riverwater flows out when the locks are closed as was the case in most of the very dry year 1972. In the valleys salinity rose from 7 to 14‰ Cl′ and in the dunes from 5 to 11‰ Cl′.

The littoral fauna extended since 1971. A freshwater fauna can now be found along the entire coast of the Haringvliet. In the westernmost part, from the dam to the island of Tiengemeten, this fauna is somewhat less rich than elsewhere, owing to the occasional influx of seawater from the sluices. In the Biesbosch a fauna of a stagnant fresh basin is gradually developing, but no Gammarids made an appearance yet in this area. Contrary to 1971 Mrs. C. H. Borghouts found *Neomysis integer* now from the sluices till the Spui.

In two fishing trips a well developed riverine freshwater fish fauna was sampled with the bottom-trawl by Dr. K. F. Vaas, consisting of roach, bream, perch and pike-perch. In March a specimen of the less frequent hybrid *Alburnus rutilo-alburnus* sensu Koumans was caught. In

the bottom samples the paucity of invertebrate animals is noteworthy. We only sampled some *Eriocheir sinensis* and some freshwater mussels: *Anodonta* and *Sphaerium corneum*.

1.3. *Grevelingen*

In this stagnant, saline basin salinity fluctuated far less than in previous year, and stayed within the limits of 16 and 17‰ Cl'. Orthophosphates fluctuated between 0.3 mg/l in autumn to 0.1 mg/l at the time of the plankton bloom. Ammonia amounted to 0.47 mg/l in winter and could not be detected in March, when the diatoms were in bloom, the species *Sceletonema costatum* attaining 15×10^6 cells per litre. In May the flagellate *Tetraselmis sp.* bloomed and flocks of *Fragillaria islandica* were very frequent.

As was the case in the former open estuary the eastern part is poorer in phytoplankton than the western part close to the dam. Zooplankton is composed of copepodes, ciliates, tintins, *Noctiluca* and larvae of *Polydora*. During the plankton blooms in March, May and June, the surface was often supersaturated with oxygen, but in some deeper parts anaerobia with production of H_2S took place. A deep pit near Den Osse was artificially destratified in 6 days by means of compressed air by the Department of Roads and Waterways. The nearly pit at Scharendijke was left undisturbed as a control. Here the anaerobia disappeared as a result of natural causes – wind and waves – within a period of 72 days. During this experiment the changes in biota were studied by Peelen and we hope to report on the results in the near future.

Primary production was measured monthly by Mr. F. Vegter by means of the 14C-method. Most curves, depicting the relationship between photosynthesis and depth show the depression near the surface caused by too much light. Before the closure this phenomenon could never be observed owing to the amount of silt suspended in the water. In March the excessive amount of plankton had the same result, at that time the inhibition at the surface was not established either. Total production integrated over the whole depth of the euphotic zone, was higher in May than in March, although the concentration of phytoplankton was higher in March. In May, however, the light penetrated further down and daylight was longer.

Around the end of September Peelen found a sudden decline in phytoplankton lasting until medio October when a gradual recovery took place. Zooplankton remained unaffected. The phenomenon was most evident near Herkingen and it was found that the water from the polders in that region proved to be highly toxic towards phytoplankton, even in dilutions of 1 : 9000. During the relevant period an agricultural spray, known as "DNBP in oil" was extensively used to destroy the leaves of potato plants in order to facilitate digging of the potatos and to combat fungi and vira. The spray destroys the chlorophyl of the leaves and is thus likely

to affect phytoplankton cells as well when it enters the basin with the water discharged by the polders.

The epilithic algal vegetation growing sublittorally changed drastically in the course of the year. The number of red and brown algae dropped to about 60% of its previous number. The ecosystem is still very young and hardly recovered from the shock of the closure. As is usually the case under such circumstances some specimens show an excessive development. This was the case here with the marine brown alga *Scytosiphon lomentaria*. *Codium fragile*, a fairly stenohaline alga, also showed a good growth as did the seagrass *Zostera marina* and the green algae *Ulva lactuca* and *Enteromorpha* spp.

On sandy places, now permanently emerged, a thin layer of blue-green algae – *Schizothrix calcicola*, *Anacystis* spp. – grew out between the sand grains just beneath the surface. With their mucous envelops these algae bind the sand grains and they enrich the soil with organic substances.

In large regions the sandy soil remained unstable during the whole year, the soil moisture remained saline and hardly any higher plants could develop. On soils with clay, where the material was not blown away, thick vegetations of *Salicornea europea* and *Suaeda maritima* were established.

On the mudflats of Flakkee *Spartina townsendii* died on the higher parts and was replaced mainly by *Suaeda maritima*. Other halophytes, such as *Halimione portulacoides* and *Puccinellia maritima* not only showed a decline but also a migration towards lower places.

In the littoral fauna few changes were observed. Some species, such as *Pycnogonum littorale* and *Buccinum undatum*, still present in 1971, could no longer be found. As no live *Littorina saxatilis* had been found 12 weeks after the closure, it was decided to test the hypothesis that the cessation of the tides would be the main cause. For this reason Mrs. Borghouts placed 100 snails in an aquarium with a simulated tidal rhythm and another 100 in a container with stagnant seawater. Both containers were constantly flushed and aerated. After five weeks only 3 *Littorina* survived in the stagnant water but 91 were still alive in the aquarium with tidal movement.

Immediately after the closure *Praunus flexuosus* had been the only opossum-shrimp still to be found in the stagnant Grevelingen basin, but during the winter of this year some *Mesopodopsis slabberi* were sampled again and since August *Neomysis integer* is also regularly encountered.

Mr. W. J. Wolff's investigations of the secundary production of the benthos were so far advanced at the end of the year that of the five most important species from six representive habitats, biomass and growth could be calculated within the limits of 95% probability. In his calculations Wolff was assisted by Mr. E. Meelis from the Institute for Theoretical Biology at Leiden and Dr. A. G. Vlasblom, working with our own Olivetti "table-computer". Observations on predation by wading birds are being analyzed. The work on flat fishes is carried on by a student from Leiden,

Mr. M. A. Mandos. It is furthermore interesting to note that some typical marine species like *Echinocardium cordatum* and *Angulus tenuis* were still alive a year after the closure.

During monthly fishing trips Vaas found the benthic fishfauna to develop along similar lines as did the fauna of Lake Veere. Here too the number of species is declining owing to the severed contact with the sea and the population of plaice is aging because the fish cannot escape to the sea and the immigration of young plaice is very small.

1.4. *Keeten, Krammer, Volkerak*

Owing to the increased influx of freshwater through the sluices in the Volkerakdam – a measure taken in order to counteract the penetration of saline water towards the north – the part of the Volkerak from the dam till the mouth of the river Dintel became mesohaline and more freshwater plankton is now encountered, originating from the Hollands Diep.

The halophytic vegetation along the shores showed the regression, already described in the previous report, to a still greater extend. Arranging the changes observed in different places according to decreasing augmentation of tidal influence, it is found that the largest augmentation is apt to induce a change from a certain vegetation towards another one belonging to another syntaxonomic alliance, but that a small augmentation will cause a change to a vegetation belonging to the same alliance.

1.5. *Oosterschelde*

The total phosphorus load of the Oosterschelde was calculated as $\pm$ 3.6 g P/m^2/yr. In spite of this heavy load the orthophosphate concentration in the mouth fluctuated only between the low values of 0.01 and 0.05 mg PO_4–P/l, as a result of the tidal influence. Nitrates fluctuated from 0.6 mg/l in February to a minimum of 0.1 mg/l in summer, and ammonia between 0.6 and practically zero sometimes in summer and autumn. Silica also showed a large fluctuation: from 1.1–1.4 mg/l SiO_4–Si/l in spring to about 0.03 mg/l in summer.

Mr. C. Bakker found phytoplankton development to be more impressive in the mouth of the Oosterschelde than in the basin, attaining a value of 0.050–0.060 extinction-units as compared to 0.030–0.035 units in the basin. The spring bloom was formed by the diatoms: *Biddulphia aurita, Thalassionema nitzschioides, Ditylium brightwellii* and *Rhizosolenia setigera.*

In summer, towards the end of August, *Biddulphia sinensis, Coscinodiscus concinnus, Triceratium alternans* and some other diatoms occurred in large numbers, together with the dinoflagellate *Ceratium fusus.* In October the smaller autumn bloom took place, caused a.o. by *Biddulphia regia* and *Ceratium fusus.* Generally speaking the mouth of the Oosterschelde contains more pelagic planktonic elements and the basin more littoral-benthic forms.

During the summer-bloom of the diatoms *Coscinodiscus concinnus* showed an impressive outburst in the coastal water outside the Oosterschelde as well. As far as the northern part of the Province Noord-Holland, the coastal seawater harboured enormous numbers of this diatom and when the cells began to decay and sink to the bottom towards the end of June, temporary oxygen depletion and foul smelling water were the results. This phenomenon had also taken place in 1964 in the Oosterschelde but was restricted to the coastal seawater in 1972.

Mr. H. A. Waardenburg, a student from Utrecht, studied the sociology of epilithic submerged algae with Scuba-techniques, assisted by divers from the institute. Algae were found down to a depth of 4 m beneath MLW. A *Laminaria saccharina* vegetation is growing down to a depth of 2 meters, *Codium fragile* down to 3.5 m and a red algal vegetation of *Ceramium rubrum* and *Polysiphonia* spp. was found to grow from over the waterline till a depth of 3.5 meter. The – in the Netherlands – rare species *Rhodochorton floridulum* could be found the whole year round but best developed in winter. Various algae with a southern distribution, such as *Dictyota dichotoma* and *Chondria dasyphylla*, are well developed from May till October.

The halophytic phanerogams growing along the shores had shown a change about the year 1965, when various species adapted to abrupt fluctuations in environmental circumstances came to the fore, such as *Suaeda maritima*, *Aster tripolium* and *Spergularia* spp. About 1970 the vegetation changed again, resuming its original character. The first change will have been the result of the construction of the secundary dam through the Grevelingen at Bruinisse increasing the influence of the riverwater, the regression of 1970 is probably a result of the closure of the Volkerak (1969) which event blocked the influence of the freshwater and caused the environment to become more saline.

Zoological investigations in the area comprised the continuation of previous studies on predation by wading birds and flat fishes on the benthic fauna. The sampling of flatfishes was severely hampered this year by excessive growth of *Ulva lactuca*, which blocked the nets. For this reason only tidal migration of the fishes could be studied.

1.6. *Veerse Meer*

This year salinity rose to high values not yet observed in previous years. The minimum was 12‰ Cl′ and the maximum 14‰.

Fluctuations in salinity were smaller than ever before: only 1.4‰ in the western part. At the spring-bloom of the phytoplankton still 0.25 mg/l PO_4–P/l could be found in the water. The maximal value, established between blooms, was 0.8 mg/l. Nitrates were totally exhausted by the phytoplankton bloom.

The tendency of the plankton already noted by Bakker, to become richer the more the salinity rises and remains contant, was seen to continue

throughout 1972. In July–August the diatom *Chaetoceros debile* established a density almost as large as other diatoms attained during the spring bloom. This did never occur before. The diatom probably was introduced, together with other cells, with the polder discharge. But, when in previous years this introduction resulted in an outburst of various μ-algae, this time the higher salinity favoured this euryhaline, marine species. The introduction of water from the Oosterschelde via the locks at Kats usually resulted in the establishment of various diatoms in the hypolimnion only, but this year many species could be found above the halocline as well. This was the case with *Rhizosolenia setigera*, *Biddulphia aurita*, and the dinoflagellate *Ceratium fusus*. In the central part of the lake various marine species were found for the first time: *Chaetoceros compressum*, *Peridinium claudicans* and *Codiella ampla*.

Beeftink found the vegetation of higher plants on the Middelplaten developing as in previous years. Species such as: *Poa pratensis*, *Cirsium arvense*, *Epilobium parviflorum* and *Agrostis stolonifera* decreased, others such as *Bellis perennis* and *Calamagrostis epigejos* increased, but the over-all result of the changes creates the impression that the future will witness a somewhat poorer vegetation than the present one.

As was the case with the plankton, the littoral fauna showed an increase in marine elements too. *Hydrobia ulvae* can again be found, whereas *Hydrobia stagnorum* is practically absent. The barnacle *Balanus amphitrite amphitrite* again enlarged its territory. Continuing her studies on shipworms Mrs. Borghouts placed small logs in the lake and regularly checked the settling of larvae in them. The main period proved to be the first two weeks of August. The benthic fishfauna was sampled monthly with a beam trawl to study the population of *Gobius niger*. At the time 4 year-classes live in the lake. Females were found to be slightly heavier than males of the same length. This year the black goby was also seen in the Canal through Walcheren and in the Oosterschelde near the sluices at Kats. The plaice is restricted to a few old individuals congregating at certain places.

In the Institute of Fisheries at Ymuiden, Mr. M. A. van Arkel, a student from Utrecht, studied herring larvae from the lake under the direction of Dr. J. J. Zijlstra, in order to decide to what race they belong. Samples taken in 1970 and 1971 were found to differ from earlier samples. As it had been established in our institute that herring is able to spawn in the lake, it was decided to study the process of hatching of herring eggs from the lake at Yerseke. Van Arkel could draw the conclusion that the herring population of lake Veere is a mixture of two races. One race, originating from the Sandettié-stock, is unable to propagate in the lake and is therefore bound to decrease in numbers, the other race, most probably originates from coastal herrings spawning in spring. This race does spawn in the lake and will become quantitatively more important in the future.

1.7. *Westerschelde*

As the Westerschelde will be the only estuary with an open connection to the sea when the dams are finished and thus a good deal of sewage is planned to be discharged in this water, it had been decided in 1969 to study the oxygen situation. Summarizing earlier data Bakker constructed the following table showing the limits between which the average amount of oxygen dissolved in the water has fluctuated in the period 1969 through 1972.

	Summer		Winter	
	mg/l O_2	% satuaration	mg/l O_2	% satuaration
Zandvliet	2 –3.5	20–40	2.5–4	20–40
Bath	3.5–5	40–60	5 –6.5	45–60
Baalhoek	5 –6	60–70	7 –8	60–70
Hansweert	6 –7	70–80	8.5–9	80–85
Terneuzen	7	80–90	8.5–9.5	85–95
Vlissingen/Breskens	8	90–100	9 –10	93–103

The poor quality of the water crossing the border and the large improvement during its course towards the sea are clearly demonstrated.

The plankton showed a strong bloom of the euryhaline, marine diatoms *Biddulphia sinensis*, the brackish diatom *Coscinodiscus jonesianes var. commutata* and the euryhaline copepode *Eurytemora affinis* in July in the brackish water region from Zandvliet to Hansweert. All species of coastal copepods living in the Oosterschelde were also found in the seaward part of the Westerschelde.

On May 25 a tanker ran aground near Rilland and part of the salt marsh near Zimmermanpolder was damaged by outflowing fuel oil. Algae living on the soil (*Enteromorpha* spp.) covered by a thin film of oil were hardly affected, but *Vaucheria* vegetations, growing along creeks and covered by an oil layer of some millimeters, suffered badly, *Vaucheria*-cushions which had been covered by a layer of $\frac{1}{2}$ millimeter were able to survive in the laboratory after some months. Generally speaking the euryeceous salt-marsh algae showed a very strong regeneration mechanism.

Halophytes reacted on the oil in different ways. Shoots of *Spartina townsendii* died but new shoots grew out from the roots afterwards, however without coming into bloom. *Scirpus maritimus* shoots grew on but failed to bloom and *Aster tripolium* only lost the lower leaves. *Triglochin maritima* survived better on lower lying spots than on higher ones. The benthic and littoral fauna did not suffer so much as many animals either live burrowed in the soil or hidden under litter and flotsam. Many juvenile crabs died.

The students from Nijmegen University J. A. A. M. Leemans and

B. A. W. Verspaendonck, who carried out an ecological and syntaxonomical study of the vegetation of Saeftinge in 1971, continued their studies at the International Training Centre in Enschede and constructed vegetation maps and maps of the intensity and consequences of grazing with the aid of aerial photographs.

Zoological investigations along the Westerschelde comprised continued sampling of zoobenthos and littoral fauna, notably on the spot where the conduit-pipe for the sewage of the Province of Brabant will be constructed. As in previous years no opossum-shrimps could be found between the Belgian frontier and Brabant, further west live *Neomysis integer* were found.

1.8. *Coastal waters*

From September on the Section for Environmental Studies of the Department of Roads and Waterways carried out a plan of taking samples along the coast from the Westerschelde till Hoek van Holland. The institute collaborated and took plankton samples with the object of comparison with the plankton of the sea-arms and estuaries. Some preliminary results can be stated. The concentration of diatoms in the mouth of the Westerschelde proved to be maximal between Cadzand and Westkapelle and was seen to decrease towards the sea as well as towards the estuary.

In front of the mouth of the Haringvliet a slight decrease in salinity could be observed as a result of the outflew of riverwater when the locks are opened. For this reason some freshwater diatoms were still found among the coastal marine ones dominating the picture.

1.9. *Inland waters*

The cursory inventory of small inland waters started some years ago was completed this year, as a general impression of the hydrobiology could be formed.

Dr. S. Parma this year took up the study of chironomids started by students some years ago. He found larvae of the types: *halophilus*, *plumosus*, *barbipes* and *salinarius* and let them hatch in the laboratory in order to identify the imagines.

The population of the Adriaanpolder was regularly sampled and proved to consist of larvae of the *halophilus*- and *plumosus*-type with a small percentage of *barbipes*-type larvae. Third, and to a lesser extent, second and fourth instar larvae were found diapauzing in winter. Measurements of the salinity just above and inside the mud showed impressive vertical gradients, probably the result of seepage. At a chlorinity of $2^0/_{00}$ in the water over the mud $10^0/_{00}$ was measured in the mud-water interphase and $17^0/_{00}$ at a depth of 6 cm in the mud. The consequences of this fact for the ecology of the chironomids are being investigated.

III. Experimental Research

1. BACTERIOLOGICAL RESEARCH

To begin his studies Mr. A. B. J. Sepers has chosen the ecology of ammonifying micro-organisms. A fairly large number of strains was isolated by plating watersamples from various estuaries directly out on media containing a mixture of amino acids and on media with a single amino acid, acting as sole source for carbon, nitrogen and energy.

At the time experiments are run in a chemostat with the object of isolating ammonifying bacteria from solutions where a special amino acid acts as the substrate limiting growth.

Mr. J. W. Rijstenbil, a student from Wageningen, started an investigation of suitable methods for chemical analysis of amino acids.

2. RESEARCH ON EURYHALINE ALGAE

The studies on the life-cycle of the green thread-alga *Rhizoclonium riparium* grown in vitro were completed. In this alga Mr. B. H. H. de Bree found an alternation between a generation of threads propagating by means of four flagellated zoo-spores and a sexual generation with gametes. Zoo-spores with 2 flagella, however, are also very frequently observed, notably in the field. Populations living under extremely adverse conditions on salt-marshes, hardly ever produce spores but propagate by means of fragmentation. Those, however, living on less dry environments such as on jetties along the sea coast and along the shores of the Oosterschelde, produce spores.

Rhizoclonium was also cultivated in the laboratory in order to test clinal variability of the genus and to observe how morphological parameters might change along a gradient from the lower until the higher parts of the littoral zone. Variation proved to be induced by the environment and not to be based on genetic difference. After a few months of cultivation a pedegree is formed characteristic for the environmental conditions at hand (long day, short day, various temperatures etc.).

As *Vaucheria* sampled in the field can only be classified when sexual organs are present, it was necessary to cultivate various samples. To date 18 different strains have been isolated.

3. INFLUENCE OF VARIOUS CULTURAL PRACTICES ON THE HIGHER VEGETATION

The plots established by Beeftink in 1971 already showed distinct reactions on the various agricultural practices applied. In the *Puccinellietum* the species *Puccinellia maritima* and *Limonium vulgare* suffered from all forms of intervention and reacted most strongly on spraying, digging and covering with soil. The latter two practices however furthered growth of *Suaeda maritima.*

In the *Halimionetum* all practices encouraged the development of *Suaeda,* mowing that of *Puccinellia maritima.*

4. PLANKTON RESEARCH

For his future work on plankton Parma has chosen the Grevelingen, where he endeavours to answer the question how the population dynamics of frequently occurring zoo-plankters will react on the environmental changes via their food. Preliminary research concerning the selection of the most suitable, representative sampling station and the selection of trustable animals, is in progress. Samples collected in autumn showed that, at that time, larvae of benthic animals, such as polychaetes, lamellibranchiates and barnacles, are much more important than holoplanktic crustaceans.

5. INFLUENCE OF TEMPERATURE AND SALINITY ON METABOLISM OF ANIMALS

Miss C. J. Smits, a Leiden student, finished her studies on *Capitella capitata*, carried out under the guidance of Vlasblom and Wolff. She found that this worm can tolerate such low salinities that this factor will not limit its distribution in most parts of the deltaic area. By means of choice experiments it was established that the animal does not prefer sands of a certain grain-size, a parameter closely correlated with the concentration of organic substance in the sand.

No correlation could be found between the number of animals per square meter and the amount of carbon in the soil. All this evidence, added to the fact that *C. capitata* is very sparely represented in the Westerschelde, one of the most polluted waters in the area, strongly indicates that this species can not be regarded as an indicator of pollution in our region.

Additional measurements of the osmotic value and the conductability of the blood of plaice from various habitats, conformed previous year's result that the total osmotic value and the level of electrolytes of plaice increases in the order Lake Veere-Grevelingen-North Sea. This increase is exclusively caused by an increase in electrolytes, the non-electrolyte component is equal for all three environments. Plaice from Lake Veere and Grevelingen showed a seasonal rhythm in their osmotic blood pressure. In winter and spring the concentration of non-electrolytes is above the annual average.

Investigations on the relationship between environmental salinity and intensity of respiration of *Neomysis integer* were continued this year.

In various combinations of the chlorinities of 0.3–0.4–0.5–2.0–4.0–8.0–12.0 and 18‰ Cl′ with 5°–10° and 20° C, no direct connection was found between the intensity of respiration and the salinity of the environment.

K. F. VAAS

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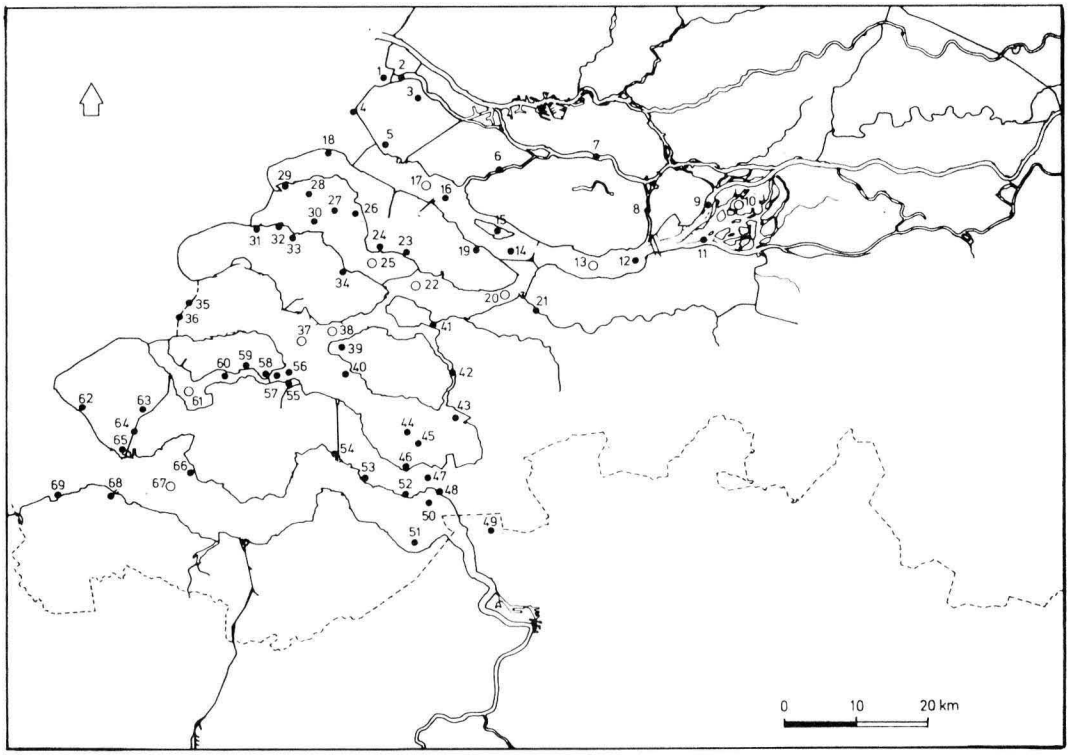

1. Brielse Gat
2. Brielse Meer
3. Brielle
4. Punt van Voorne
5. Quack
6. Spui
7. Oude Maas
8. Dordtse Kil
9. Nieuwe Merwede
10. Biesbosch
11. Amer
12. Sasseplaat
13. Hollands Diep
14. Ventjagersplaat
15. Tien Gemeten
16. Slijkplaat
17. Haringvliet
18. Kwade Hoek
19. Den Bommel
20. Volkerak
21. Dintel
22. Krammer
23. Battenoord
24. Herkingen
25. Grevelingen
26. Slikken van Flakkee
27. Veermansplaat
28. Hompelvoet
29. Springersgors
30. Stampersplaat
31. Scharendijke
32. den Osse
33. Brouwershaven
34. Dijkwater
35. Neeltje Jansplaat
36. Noordeiland
37. Oosterschelde
38. Keeten
39. Stavenisse
40. Dortsman
41. Schelde–Rijn canal
42. Eendracht
43. Bergse Diep
44. Lodijkse Gat
45. Pietermanskreek
46. Stroodorpepolder
47. Rilland
48. Bath
49. Zandvliet
50. Nauw van Bath
51. Saaftinge
52. Zimmermanpolder
53. Waarde
54. Hansweert
55. de Rietput
56. Zandkreek
57. Katse Plaat
58. Katseveer
59. Adriaanpolder
60. Middelplaten
61. Veerse Meer
62. Zoutelande
63. Middelburg
64. Canal through Walcheren
65. Vlissingen
66. Kaloot
67. Westerschelde
68. Breskens
69. Verdronken Zwarte Polder