# Acta Medica Hungarica

VOLUME 41, NUMBER 1, 1984



Akadémiai Kiadó, Budapest

ACTA MED HUNG. 41(1) 1–73 (1984) HU ISSN 0236–5286

# ACTA MEDICA HUNGARICA

A QUARTERLY OF THE HUNGARIAN ACADEMY OF SCIENCES

*Acta Medica* publishes reviews and original papers on clinical and experimental medicine in English.

*Acta Medica* is published in yearly volumes of four issues by

AKADÉMIAI KIADÓ

Publishing House of the Hungarian Academy of Sciences
H-1054 Budapest, Alkotmány u. 21

Manuscripts and editorial correspondence should be addressed to the Managing Editor:

Dr. Miklós Papp
*Acta Medica*
H-1083 Budapest, Szigony u 43 or H-1450 Budapest 9, P. O. Box 67

*Subscription information*

Orders should be addressed to

KULTURA Foreign Trading Company
H-1389 Budapest, P. O. Box 149

*Acta Medica is indexed in Current Contents*

# ACTA MEDICA HUNGARICA



VOLUME 41

AKADÉMIAI KIADÓ, BUDAPEST
1984

## CONTENTS

### NUMBER 1



### NUMBERS 2—3

## NUMBER 4

THEMES

# AUTHOR INDEX

## SUBJECT INDEX

## CONTENTS

ENDOCRINOLOGY

*Acta Medica Hungarica, 41 (1), pp. 3—6 (1984)*

# COMMEMORATION OF THE TENTH ANNIVERSARY OF THE DEATH OF PROFESSOR MIKLÓS JULESZ

F. A. László

Received: February 28, 1983

It is now 10 years since the death of Professor Miklós Julesz, Director of the First Department of Medicine at the University Medical School in Szeged, Hungary.

He was appointed to lead the Department in 1959, under difficult conditions. As a consequence of the events in 1956, several of the staff had left the Department, and even the country. Further, it was necessary for him to follow in the footsteps of such famous predecessors as István Rusznyák and Géza Heténvi. On his arrival, Professor Julesz expressed his hopes that, in spite of the losses, all of the valuable features of the department would be preserved. Now, more than 20 years later, it can be said categorically that his hopes in this respect were fully realized. He not only maintained the high standard of the clinical work, but instituted further substantial developments. I shall mention only two examples: in 1960 he created the now independently functioning Endocrine Unit, and in 1962, the Isotope Laboratory.

Send offprint requests to F. A. László 6701 Szeged P.O.B. 469, Hungary

Miklós Julesz was a physician, a pedagogue and a medical researcher, but at the same time he was very active in public affairs.

He can be regarded as the founder of clinical endocrinology in Hungary. He was a physician with great experience, with a good power of observation and wide-ranging knowledge, and an excellent organizer. He had a great depth of feeling for his patients, dealing not only with the disease, but also with the human individual. His attitude to the patients was characterized by a profound humanism, which he epxressed as "The patient is always right". Indeed, under all circumstances he remained faithful to the patients, and they remained faithful to him.

His powers of observation and his excellent theoretical knowledge were accompanied by a most up-to-date versatility in biochemistry. These features led him in 1957 to make use of his literary background and the abundant casuistic material by writing a much needed monograph "The pathology and diagnostics of neuroendocrinological diseases". This book proved of great help to general practitioners and internists, and for a long period it was the standard work of reference for endocrinologists. This important handbook was followed in 1966 by the monograph "The treatment of endocrine diseases and its theoretical basis" that can be regarded as the fundamental handbook of Hungarian clinical endocrinology.

The thoroughness of his clinical observations and his ability to discern relationships were exemplified by his descriptions of new diseases. He outlined the essence of secondary Cushing's disease and the secondary Morgagni syndrome, and he described the clinical picture of basophilism in puberty, which was later named after him.

He very much liked to teach; he considered it an essential part of the duties of a university professor. He prepared seriously and thoroughly every lecture he delivered. He kept careful notes, and supplemented them with recent data before his lectures. His enthusiastic pedagogic work did not extend only to the endocrine diseases; he enjoyed lecturing also on the circulation and on renal diseases. He dealt in an exemplary manner with the students and qualified doctors alike. As a consequence of his material and spiritual support of the activities of his young colleagues he succeeded in creating clinical endocrine schools in both Budapest and Szeged and these schools have continued to thrive.

A close study of the two monographs mentioned above clearly demonstrates that his clinical research work covered virtually the whole of endocrinology: he dealt with the normal and pathological functioning of the thyroid, the parathyroid, the adrenals and the gonads, just as with the functions of the diencephalon and the pituitary. Of his numerous important observations only a few need to be mentioned. He showed that ketosis was a strong stimulus of the function of the basophilic cells of the pituitary. He described the effect

of vitamin $B_1$ on the function of the adenohypophysis; the experience stemming from this observation is still used in clinical practice. He observed the beneficial effect of hyaluronidase in the treatment of exophthalmos of thyroid origin. He was among the first to emphasize the significance of the periphery in endocrinology. He spoke on this topic in his inaugural lecture when appointed to the Chair in Szeged. The concept described 23 years ago has proved an extremely fruitful one. Its validity is demonstrated by the fact that research of the periphery is at present one of the most promising branches of experimental endocrinology. A product of this research attitude and his studies with biochemists was his next monograph "Steroids in human skin". In this book he and his colleagues were the first to describe the importance of one of the large peripheral organs, the skin, in androgen steroid metabolism. They showed that important androgen metabolism processes take place in human skin. Their work led to an entirely new approach to the pathogenesis of hirsutism. Among others, they proved that androgen production in the skin is considerably enhanced in cases of idiopathic hirsutism, exhibiting normal systemic androgen conditions.

Even if his work could be regarded as basic research, it always had the aim of answering questions raised at the patient's bedside. He continued and further developed the concepts applied by Rusznyák and Heténvi: in the university departments it is not only possible, but necessary to carry out basic research, with the organization of appropriately qualified research groups who, cooperating with the clinicians, attempt at solving problems posed by life and practice.

More than 20 years ago, Professor Julesz clearly saw that fundamental results in endocrinology can only be achieved by organized research cooperation between chemists, biochemists, physiologists, pathophysiologists and naturally clinicians. This guiding principle and the attainment of the appropriate conditions led in 1978 to the development in Szeged of a coordinated endocrine research programme with the participation of four research groups (Departments of Medical Chemistry, Pathophysiology, Internal Medicine and Gynaecology), with the central support of the Hungarian Academy of Sciences. Even on a national scale, the effectiveness of the new type of organization is well recognized and is considered to be exemplary.

Professor Julesz understood that, from the aspects of endocrine research, the necessity for development within Hungary must be accompanied by international recognition. He therefore organized the First Hungarian Endocrine Symposium in 1958, and made considerable efforts towards the foundation of the Hungarian Endocrine and Metabolism Society, of which he later became the first Chairman, remaining in this post until his death.

The activities of Professor Julesz received state recognition with his award of the Kossuth Prize. He was also elected a Corresponding Member of the Hungarian Academy of Sciences.

At the end of my commemoration I should like to speak of Miklós Julesz as a man, as a leader, and as a chief.

His whole life was characterized by his wide-ranging erudition, by his passionate love for literature and music, by his deep humanism and by his permanent optimism. In physique he was not strongly built, but he was nevertheless a strong man. In the course of his life he underwent severe ordeals, but he remained a human even under inhuman conditions. It was his unshakable faith that helped him to return from "the depths of hell", as he referred to his experiences in Buchenwald.

He helped his colleagues from a professional aspect, but they could also turn to him at any time with their personal problems. It was his guiding principle that his young colleagues must not only be taught, but also educated, to accept their responsibilities, to be upright, and to be irreproachable in their research. The most powerful means that he used for this purpose was his own personal example. He lived in accordance with strict medical ethical demands and in an exemplary family environment. With strong will-power, a clear conception of the goal and limitless diligence, from day to day he strived to achieve ever more results.

Characteristic features of his leadership were his quiet voice, but at the same time his resolution. He never attempted to monopolize the department. He always avowed, and in practice too adhered to the principle that the best functioning department in present age is one with several profiles. He not only preserved the existing clinical profiles and ensured the suitable balance between them, but also assisted their development. He built up his own research group without haste or aggression; the basis was always willingness, interest and enthusiasm. This played a part in the consequence that his research team proved a durable and effective one, and is still operating well even 10 years after his death.

Life is fleeting, but life goes on in the following generation of children and students. Such an occasion as this one is always sad, for we all remember what we have lost; we must, however, remain optimists if the work that has begun continues to flourish.

The activities of Professor Julesz were worthy of his great predecessors: he not only carried on the traditions of Rusznyák and Heténvi, but raised these to higher levels. He maintained the existing values and created new schools of endocrinology in both Budapest and Szeged.

Perhaps a fitting way to commemorate the figure of Miklós Julesz is to present some of the most recent scientific results of his school to all those of you who have joined us to pay tribute to him on this occasion.

*Acta Medica Hungarica, 41 (1), pp. 7—18 (1984)*

# METABOLISM AND CONCENTRATION OF ANDROGENIC STEROIDS IN ABDOMINAL SKIN OF HIRSUTE WOMEN WITH ANDROGENITAL SYNDROME

I. Tóth and I. Faredin

ENDOCRINE UNIT AND RESEARCH LABORATORY, FIRST DEPARTMENT OF MEDICINE, UNIVERSITY MEDICAL SCHOOL, SZEGED, HUNGARY

Received: February 28, 1983

An in vitro study has been made of the steroid metabolism in the abdominal skin in two hirsute women with adrenogenital syndrome, and the concentrations of the various steroids in the skin tissue have been determined. The urinary excretion of the total 17-Ks, and particularly P-triol, was pathologically high, while of the androgens examined in the serum, primarily the levels of $\Delta^4$-dione and Test, were found to be elevated. The 21-hydroxylase deficiency meant that the plasma ACTH level was likewise extremely high in both patients.

In vitro incubation studies demonstrated that in one patient (with the higher androgen overproduction) more Test. than normal was formed from the precursors (DHA, $\Delta^5$-diol, $\Delta^4$-dione), i.e. the biosynthetic pathway (17$\beta$-HSD, $\Delta^5$-3$\beta$-HSD) leading towards the androgens was enhanced in the abdominal skin. In the other patient (where the androgen production was less high as a consequence of the earlier adrenalectomy) the metabolism in the abdominal skin was not enhanced; indeed, for many metabolites the extent of the transformation did not even attain the level for normal women. The activity of Test. 5$\alpha$-reductase was not increased in the skin of either patient.

The results on the steroid contents of the skin tissue revealed that numerous free steroids (DHA, And., $\Delta^4$-dione, $\Delta^5$-diol, Test., DHT) and $C_{19}$-steroid sulphates were present in higher concentrations than in the abdominal skin of healthy women. The extents of steroid accumulation compared to the serum level in the same patient were pathologically high in the case of $\Delta^5$-diol, DHT and DHA-S in the abdominal skin of the two hirsute women with adrenogenital syndrome. This confirmed that a state of hyperandrogenism does exist in the skin of these patients.

**Keywords:** adrenogenital syndrome, steroid metabolism, skin steroids

## Introduction

Hirsutism is the pathological growth of hair in females, importance being attributed in its development to the skin, and within this to the hair follicles, as target organs. The main stimulants of the growth of hair in both males and

Send offprint requests to I. Tóth H-6701, Szeged, P.O.B. 469, Hungary

The following abbreviations have been used: 17-Ks = 17-ketosteroid; 17-OH-CS = 17$\alpha$-hydroxycorticosteroid; P-diol = 5$\beta$-pregnane-3$\alpha$,20$\alpha$-diol; P-triol = 5$\beta$-pregnane-3$\alpha$,17$\alpha$, 20$\alpha$-triol; 5$\alpha$-ane-dione = 5$\alpha$-androstane-3,17-dione; $\Delta^4$-dione = 4-androstene-3,17-dione; And. = 3$\alpha$-hydroxy-5$\alpha$-androstane-17-one; $\Delta^5$-diol = 5-androstene-3$\beta$,17$\beta$-diol; DHA = 3$\beta$-hydroxy-5-androstene-17-one; DHT = 17$\beta$-hydroxy-5$\alpha$-androstane-3-one; Test. = 17$\beta$-hydroxy-4-androstene-3-one; Epiand. = 3$\beta$-hydroxy-5$\alpha$-androstane-17-one; And.-S = 3$\alpha$-sulphooxy-5$\alpha$-androstane-17-one; DHA-S = 3$\beta$-sulphooxy-5-androstene-17-one; $\Delta^5$-diol-S = 5-androstene-3$\beta$,17$\beta$-diol-3-sulphate; Test.-S = 17$\beta$-sulphooxy-4-androstene-3-one.

females are known to be the androgenic steroids (testosterone, 5α-dihydrotestosterone). At the time of the leadership of Professor Julesz, we set out from these considerations and put forward a working hypothesis: all human skin has a definite steroid turnover, the composition and quantitative relations of which are determined jointly by the androgenic steroids passing into the skin with the blood stream, and by those formed locally in the skin [14]. On this basis, hirsute females may be divided into two main groups:

(a) hirsutism of known origin, where the adrenal cortex or the ovary is responsible for the overproduction of androgen, and

(b) idiopathic hirsutism of unknown origin, where the androgen level of the peripheral blood is normal.

This paper presents our results relating to the steroid metabolism in females with hirsutism of adrenocortical origin, and to the hyperandrogenism of the skin.

## Materials and methods

The chemicals and solutions used in the investigations were of analytical purity. The organic solvents were purified in accordance with described procedures, and were then subjected to fractional distillation [14].

1. *Radioactive steroids:* The [4-$^{14}$C]-labelled dehydroepiandrosterone (DHA; specific activity [S.A.] = 57.1 mCi/mmol), 5-androstene-3β,17β-diol ($\Delta^5$-diol; S.A. = 57.1 mCi/mmol), 4-androstene-3,17-dione ($\Delta^4$-dione; S.A. = 56.6 mCi/mmol) and testosterone (Test.; S.A. = 22.19 mCi/mmol), and the [$^3$H]-labelled [7-$^3$H(N)]DHA (S.A. = 24 Ci/mmol), [1,2(N)-$^3$H]-dihydrotestosterone (DHT; S.A. = 60 Ci/mmol) and [1β,2β-$^3$H]Test. (S.A. = 44.6 Ci/mmol) were products of the Radiochemical Centre (Amersham, England). The [7-$^3$H(N)]$\Delta^5$-diol (S.A. = 20 Ci/mmol), [1,2-$^3$H(N)]androsterone (And.; S.A. = 40 Ci/mmol) and the ammonium salt of [7-$^3$H(N)]DHA-sulphate (DHA-S; S.A. = 24 Ci/mmol) were bought from New England Nuclear (Boston, U.S.A.).

Before use, these radioactive steroids were purified by column and thin-layer chromatography [14, 19].

2. *Steroids in the urine and the serum* were determined as described previously [5, 6, 22].

3. *Patients.* An account will be given of the examinations on two hirsute women from among the adrenogenital syndrome in-patients of the Endocrine Unit. An intensive growth of hair could be observed all over the body of the 18-year-old R. A. and the 34-year-old O. S., particularly on the face, arms and chest. The enlarged clitoris had been amputated in both cases, while the left adrenal had been removed from the 34-year-old patient at the age of 12 years in an attempt to lower the hormone level.

In both patients the urinary total 17-ketosteroids, the 17-ketosteroid fractions, and particularly the pregnanetriol, were pathologically high (Table I).

Of the androgens examined in the serum (Table II), especially the levels of $\Delta^4$-dione and the Test. formed from it were pathologically elevated. The plasma ACTH level was extremely high in both patients (79 and 40.6 pmol/l; normal range 8.8—26 pmol/l).

The diagnosis of adrenogenital syndrome was established on the basis of the clinical picture, gynaecological examinations and the laboratory results. Skin samples were excised by a surgeon from the suprapubic hair-covered abdomen.

4. *Incubation of skin slices.* In vitro incubation studies were carried out to establish whether the activities of the enzymes metabolizing the androgens are changed in the abdominal skin of women with hirsutism of adrenocortical origin. A 0.5 g portion of the excised skin was cut into 1—2 mm slices, which were incubated with various [4-$^{14}$C]-labelled steroids in the presence of coenzymes in KRPG medium [4, 7, 8, 9, 14]. The metabolites produced were identified, and their percentage conversions were determined. Conclusions were drawn as to the activities of the individual enzymes from the percentage transformations.

**Table I**

*Urinary steroid excretion in hirsute women with adrenogenital syndrome*

| Patients | Total 17-Ks (μmol/d) | Total 17-OH-CS (μmol/d) | P-triol (μmol/d) |
|---|---|---|---|
| R. A. 18 years | 600 | 25.4 | 241 |
| | 510 | 23.7 | 199 |
| | 413 | 21.2 | |
| O. S. 34 years | 63.5 | 1.9 | 71 |
| | 103.7 | 2.8 | 119 |
| | 140.9 | 6.6 | 125 |
| Normal range: | 17.4—52.0 | 5.0—16.6 | 1.2—7.7 |

*17-Ks fractions (μmol/d)*

| | *R. A. 18 years* | *O. S. 34 y.* | *Normal range:* |
|---|---|---|---|
| DHA | 83.6 | 15.3 | 4.8—15.3 |
| And. | 207.1 | 47.5 | 7.3—14.2 |
| Etio. | 105.1 | 17.0 | 8.7—17.0 |
| 11-oxyd.-And | 44.1 | 14.6 | 0.5—3.5 |
| 11-oxyd.-Etio. | 84.7 | 21.2 | 1.2—4.5 |

**Table II**

*Serum androgen levels in hirsute women with adrenogenital syndrome (nmol/l)*

| | | $\Delta^4$-dione | $\Delta^5$-diol | Test. | DHT |
|---|---|---|---|---|---|
| Patients | R. A. 18 years | 55.59 | 8.19 | 18.22 | 2.37 |
| | O. S. 34 years | 13.78 | 7.08 | 12.49 | 2.17 |
| Healthy women | mean | 6.28 | 3.65 | 2.39 | 2.17 |
| (18—46 years) | range | 2.97—9.36 | 2.10—5.61 | 0.87—3.29 | 0.34—3.79 |

5. *Study of steroid content of skin tissue.* In parallel with the incubation a 1 g portion of the excised abdominal skin was used to determine the concentrations of the free androgenic steroids (DHA, And., $\Delta^4$-dione, $\Delta^5$-diol, Test., DHT) and the more important $C_{19}$-steroid sulphates (And.-S, DHA-S, $\Delta^5$-diol-S, Test.-S [11, 24].

The skin sample was cut into 15—20 μ slices at —20°C in a cryostat, and the slices were then soaked in a 15 : 10 mixture of 2 N $NH_4OH$ and diethylether. Subsequently, the skin

tissue residue was extracted with 4×5 ml methanol while being strongly triturated. The [$^3$H]- labelled forms of the examined steroids were used as internal standards.

After halfsaturation with $(NH_4)_2SO_4$, the aqueous phase was extracted with ethyl acetate, and the extract was then combined with the dry residue from the organic solvents. From the resulting "crude extract", the free and the sulphate ester fractions were separated on a florisil column [19]. The fraction of free steroids was chromatographed on an $Al_2O_3$ column, and the individual steroids were next isolated by thin-layer chromatography [14]. The 17$\beta$-OH-steroids were measured directly, and the 17-ketosteroids after reduction with $NaBH_4$ and subsequent thin-layer chromatographic purification, by protein-binding assay (PBA) or radioimmunoassay (RIA) [22].

After solvolysis, the sulphate ester steroids were chromatographed and measured similarly as for the free androgens.

The results are expressed in units of nmol/kg wet skin tissue.

6. *Measurement of $C_{19}$-steroid sulphates in serum.* Blood samples were taken between 8 and 9 a.m., and the concentrations of DHA-S, And.-S, $\Delta^5$-diol-S and Test.-S in the serum were measured with the previously described method [20] or with the procedure used in the determination of the $C_{19}$-steroid sulphates of human hair and axillary sweat [21, 23, 25] by means of P.B.A.

## Results

### "*In vitro*" *metabolism of free androgens in abdominal skin*

From the quantities of metabolites formed during the incubation studies with abdominal skin slices from the two hirsute women with adrenogenital syndrome, conclusions were drawn as to the activities of the enzymes catalyzing the transformations. The percentage transformations referred to the radioactive substrate were compared with the corresponding values for normal, healthy women.

When the abdominal skin slices from these two hirsute women were incubated with [4-$^{14}$C]DHA (Table III) the skin of the 18-year-old patient synthetized Test. and the androgenic 17-ketosteroids (And., $\Delta^4$-dione and 5$\alpha$--ane-dione) with pathologically high percentage conversions, while only the formation of 5$\alpha$-ane-dione was enhanced for the 34-year-old woman.

As compared with the corresponding data for healthy women, from [4-$^{14}$C]$\Delta^5$-diol (Table IV) elevated amounts of Test. and And. were produced by the skin of the first patient, and of DHT by the skin of the second patient. The other metabolites were formed in lower than normal percentages in the case of the second patient (O. S.).

The skin slices from the 18-year-old patient converted [4-$^{14}$C]$\Delta^4$-dione (Table V) to Test. and to androgenic 17-ketosteroids (And., 5$\alpha$-ane-dione) in higher percentages than for normal women, while in the case of the 34-year-old patient only 5$\alpha$-ane-dione was produced in pathologically high amount, all of the other metabolites being produced within the normal limits.

When [4-$^{14}$C]Test. (Table VI) was incubated with the abdominal skin slices from these two patients, in the first case And. and 5$\alpha$-ane-dione were formed in higher quantities, whereas for the other compounds the conversion

**Table III**

*The in vitro metabolism of [4-$^{14}$C] dehydroepiandrosterone by human skin*

| [4-$^{14}$C]DHA | Healthy women 17—45 years | Hirsute women with adrenogenital syndrome | | Healthy men 19—46 years |
|---|---|---|---|---|
| | | 18 years | 34 years | |
| | conversion % | | | |
| Test. | 0.20 (0.08—0.33) n=3 | 1.62 | 0.30 | 0.48 (0.04—1.38) n=6 |
| $\Delta^5$-diol | 1.32 (1.03—1.90) n=3 | 1.18 | 1.20 | 1.85 (1.19—2.97) n=5 |
| And. | 2.14 (0.81—3.70) n=4 | 5.74 | 1.92 | 2.53 (0.62—6.84) n=6 |
| $\Delta^4$-dione | 5.34 (0.85—13.00) n=7 | 28.84 | 9.73 | 6.00 (1.37—12.39) n=9 |
| 5α-ane-dione | 1.76 (0.85—2.22) n=3 | 5.70 | 4.80 | 1.86 (0.30—4.84) n=5 |

**Table IV**

*The in vitro metabolism of [4-$^{14}$C]5-androstene-3β,17β-diol by human skin*

| [4-$^{14}$C]$\Delta^5$-diol | Healthy women (22—46 years) n=3 | Hirsute women with adrenogenital syndrome | | Healthy men (19—45 years) n=6 |
|---|---|---|---|---|
| | | 18 years | 34 years | |
| | conversion % | | | |
| Test. | 18.73 (13.2—26.8) | 28.73 | 11.81 | 8.75 (4.06—15.13) |
| DHT | 0.67 (0.40—0.98) | 0.78 | 1.01 | 1.31 (0.30—2.72) |
| And. | 0.57 (0.52—0.66) | 0.83 | 0.27 | 0.41 (0.28—0.54) |
| $\Delta^4$-dione | 5.23 (1.40—11.16) | 1.79 | 0.43 | 1.41 (0.45—4.08) |
| 5α-ane-dione | 0.53 (0.34—0.64) | 0.64 | 0.30 | 0.65 (0.30—1.86) |

was normal (Epiand., $\Delta^4$-dione) or lower than normal (DHT). For all of the metabolites, the skin of the 34-year-old patient metabolized the radioactive testosterone with lower than normal percentages.

**Table V**

*The in vitro metabolism of [4-$^{14}$C]4-androstene-3,17-dione by human skin*

| [4-$^{14}$C]$\Delta^4$-dione | Healthy women (26—43 years) n=7 | Hirsute women with adrenogenital syndrome | | Healthy men (19—37 years) n=5 |
|---|---|---|---|---|
| | | 18 years | 34 years | |
| | conversion % | | | |
| Test. | 1.80 (1.33—2.28) | 3.55 | 1.79 | 1.96 (0.91—2.62) |
| DHT | 0.37 (0.10—0.56) | 0.21 | 0.35 | 0.54 (0.18—0.86) |
| And. | 8.34 (2.57—11.42) | 25.90 | 11.32 | 10.82 (7.09—15.87) |
| Epiand. | 6.01 (1.26—10.30) | 13.55 | 2.82 | 2.39 (0.98—4.85) |
| 5α-ane-dione | 10.85 (4.2—16.14) | 17.57 | 23.40 | 12.93 (9.15—15.45) |

**Table VI**

*The in vitro metabolism of [4-$^{14}$C]testosterone by human skin*

| [4-$^{14}$C]Test. | Healthy women (26—43 years) n=5 | Hirsute women with adrenogenital syndrome | | Healthy men (19—37 years) n=5 |
|---|---|---|---|---|
| | | 18 years | 34 years | |
| | conversion % | | | |
| DHT | 5.61 (3.70—7.21) | 2.73 | 1.86 | 6.39 (3.84—9.33) |
| And. | 0.83 (0.61—1.36) | 2.17 | 0.33 | 1.37 (0.57—2.69) |
| Epiand. | 0.69 (0.33—1.59) | 1.42 | 0.12 | 0.71 (0.24—1.25) |
| $\Delta^4$-dione | 3.52 (1.82—5.11) | 2.94 | 1.41 | 4.20 (3.43—6.30) |
| 5α-ane-dione | 0.81 (0.50—1.14) | 1.76 | 0.47 | 1.22 (0.50—1.78) |

*Concentrations of androgens and $C_{19}$-steroid sulphates in abdominal skin tissue*

Since our examinations relating to the steroid content of the skin tissue have not yet been reported so widely, we shall first present our results for healthy individuals.

Of the free steroids measured in the abdominal skin tissue of healthy women and men (Table VII), DHA is to be found in the highest amount (mean ± S. E. M.: 79 ± 29, and 165 ± 78 nmol/kg, respectively). Whereas And. occurs in almost the same quantities in the abdominal skin of women and men (55 ± 19, and 48 ± 25 nmol/kg), the concentration of $\Delta^4$-dione is substantially higher in the male abdominal skin (101 ± 36 nmol/kg) than in that of women (36 ± 8 nmol/kg). $\Delta^5$-diol, Test., and DHT are to be found in decreasing amounts in the abdominal skin, but all three androgen-active steroids are more concentrated in men.

**Table VII**

*Androgenic steroids in abdominal skin tissue from healthy women and men and hirsute women with adrenogenital syndrome (nmol/kg wet weight)*

| | | DHA | And. | $\Delta^4$-dione | $\Delta^5$-diol | Test. | DHT |
|---|---|---|---|---|---|---|---|
| Healthy women | mean | 79 | 55 | 36 | 30 | 17 | 9 |
| (28—46 years) | ±S.E.M. | ±29 | ±19 | ±8 | ±14 | ±6 | ±4 |
| (n=7) | | | | | | | |
| Healthy men | mean | 165 | 48 | 101 | 35 | 35 | 21 |
| (28—47 years) | ±S E.M. | ±78 | ±25 | ±36 | ±8 | ±9 | ±9 |
| (n=5) | | | | | | | |
| R. A. 18 years | | 731 | 132 | 280 | 295 | 148 | 73 |
| O. S. 34 years | | 206 | 11 | 151 | 131 | 61 | 73 |

We next compare the steroid quantities found in the abdominal skin tissue from the two adrenogenital syndrome patients with the corresponding values for healthy women. The concentrations of all of the free steroids (particularly in the case of the 18-year-old patient) far exceed the skin steroid levels for normal women.

In the abdominal skin of the 34-year-old patient, only the And. content was lower than the value for healthy women, the levels of the other androgenic steroids were considerably in excess of the corresponding values for healthy women. It is noteworthy that in the abdominal skin from both patients the DHT was more than 8 times as concentrated as in normal women.

Of the $C_{19}$-steroid sulphates (Table VIII) And.-S occurs in the highest amount in the skin of women (132 ± 29 nmol/kg), whereas DHA-S does so in the abdominal skin of men (2162 ± 406 nmol/kg). $\Delta^5$-diol-S and Test.-S are found in lower concentrations in the skin tissue of both women and men, but here too the difference between the sexes can be demonstrated.

**Table VIII**

*$C_{19}$-steroid sulphates in abdominal skin-tissue from healthy women and men and hirsute women with adrenogenital syndrome (nmol/kg wet weight)*

| | | And-S | DHA-S | $\Delta^5$-diol-S | Test-S |
|---|---|---|---|---|---|
| Healthy women | mean | 132 | 82 | 42 | 14 |
| (28—46 years) | ±S.E.M. | ±29 | ±27 | ±7 | ±3 |
| (n=7) | | | | | |
| Healthy men | mean | 204 | 2162 | 132 | 22 |
| (28—47 years) | ±S.E.M. | ±64 | ±406 | ±41 | ±10 |
| (n=5) | | | | | |
| R. A. 18 years | | 839 | 2391 | 48 | 11 |
| O. S. 34 years | | 46 | 136 | 4 | 7 |

Of the $C_{19}$-steroid sulphates And.-S and DHA-S were extremely high in the abdominal skin of the first patient (839 and 2391 nmol/kg). The concentrations of the other steroid sulphates ($\Delta^5$-diol-S, Test.-S) in the skin did not differ appreciably from the normal values for women. In the skin of the other patient the levels of And.-S and DHA-S were substantially lower (about one-twentieth). However, as we shall see later, when these values are compared with the corresponding serum concentrations, the large differences between the two patients disappear.

## Discussion

It is generally known that the most frequently occurring enzyme defect in the adrenal cortex, the lack of or the decreased activity of 21-hydroxylase, is responsible for the androgen overproduction in adrenogenital syndrome. As a consequence of a 21-hydroxylase or 11$\beta$-hydroxylase insufficiency, the production of the corticosteroids is hindered; this leads to an enhanced ACTH secretion, and hence an excess of steroid precursors arises [13, 16]. Extensive steroid analyses on the urine and plasma suggest that the formation of a large quantity of Test. (resulting in hyperandrogenism) proceeds via the 3-keto-$\Delta^4$ precursors (progesterone → 17$\alpha$-OH-progesterone → $\Delta^4$-dione). This pathway is confirmed by the pathologically enhanced excretion of the metabolites of progesterone and 17$\alpha$-OH-progesterone (P-diol and P-triol) in the urine, and by the elevated plasma levels of $\Delta^4$-dione and Test. [15, 18].

Earlier skin incubation studies in our laboratory [4,7 — 9,14] proved that healthy female and male abdominal skin contains all those enzyme systems ($\Delta^5$-3$\beta$-HSD, 3$\alpha$-HSD, 17$\beta$-HSD, $\Delta^4$-5$\alpha$-R) which catalyze the transformations of the $C_{19}$-steroids. We demonstrated that human skin is not only capable

of transforming Test. to DHT, as studied in detail by other authors [17, 26], but is also able to synthetize Test. from the weakly androgenic precursors (DHA, $\Delta^4$-dione, $\Delta^5$-diol). Thus, the possibility exists for these androgens to form locally in the skin itself, and if these transforming enzymes display a higher than normal activity around the hair follicles, the enhanced peripheral androgen biosynthesis may lead to hirsutism even though the plasma androgen level is normal.

How do the activities of the enzyme systems participating in the skin steroid metabolism change in adrenogenital syndrome, when the androgen overproduction by the pathologically functioning adrenal cortex is responsible for the hirsutism and the virilization? Our in vitro incubation investigations showed that in the case of the first patient (R. A.), where the androgen production was substantially higher, more Test. than normal was formed from the precursors (DHA, $\Delta^5$-diol, $\Delta^4$-dione), i.e. the biosynthetic pathway (17$\beta$-HSD, $\Delta^5$-3$\beta$-HSD) leading towards the androgens was enhanced. In the other patient (O. S.), where the androgen production was less high as a consequence of the earlier adrenalectomy, the metabolism was not enhanced; indeed, for many metabolites the extent of the transformation did not even attain the level for normal women. It is interesting that the activity of the Test. 5$\alpha$-R enzyme was not increased in the abdominal skin of either patient, similarly as found by Bouchard et al. [1] in the pubic skin of hirsute women with a 21-hydroxylase insufficiency of complete type. As concerns the other enzyme systems, the differences in activity found in the abdominal skin of the two adrenogenital syndrome patients may be explained by the considerably different androgen concentrations measured in the peripheral blood and in the skin, but the role of their different ages cannot be excluded.

From the results relating to the steroid content of the skin tissue, it emerges that numerous free (non-conjugated) steroids and $C_{19}$-steroid sulphates are to be found in higher concentration in the adrenogenital cases than in the skin of healthy women. The extent of steroid accumulation in the skin can be better expressed, however, if the skin steroid quantities found in the individual cases are compared with the serum levels in the same patient. Accordingly, for all of the steroids examined, the values measured in the skin (nmol/kg) were divided by the level of the corresponding steroid in the serum (nmol/l); the steroid-accumulating ability of the skin (as androgen target organ) may be better approximated to with the resulting quotient [12].

For the free steroids (Table IX), this quotient is in all cases a number larger than one (the mean values for normal women lie in the range 4—8), i.e. the concentration of each of the androgenic steroids is higher in the skin tissue than in the blood. The quotients of $\Delta^5$-diol and DHT in the skin of the two adrenogenital syndrome patients i.e. their relative concentrations, are far higher than the corresponding values for healthy women.

**Table IX**

*Correlation between androgenic steroid contents in abdominal skin tissue and in serum of healthy women and men and hirsute women with adrenogenital syndrome*

$$\text{Ratio} = \frac{\text{steroid conc. (nmol/kg) in abdominal skin tissue}}{\text{steroid conc. (nmol/l) in serum}}$$

| | | $\Delta^4$-dione | $\Delta^5$-diol | Test. | DHT |
|---|---|---|---|---|---|
| Healthy women | mean | 5.7 | 8.1 | 7.1 | 4.1 |
| (28—46 years) | range | 3.8—12.1 | 5.3—14.3 | 5.2—19.5 | 2.4—26.5 |
| n=7 | | | | | |
| Healthy men | mean | 23.0 | 6.9 | 2.0 | 7.0 |
| (28—47 years) | range | 14.3—32.2 | 4.7—13.6 | 1.2—3.7 | 4.8—10.7 |
| n=5 | | | | | |
| R. A. 18 years | | 5.0 | 36.0 | 8.1 | 30.8 |
| O. S. 34 years | | 10.9 | 18.5 | 4.9 | 33.6 |

There is an interesting correlation between the abdominal skin tissue and the serum in the case of the $C_{19}$-steroid sulphates (Table X). Here, the value of the quotient (with the exception of Test.-S) is substantially less than one (0.01—0.1), which means that under normal conditions the sulphate ester steroids are to be found in 10—100 times higher concentrations in the blood than in the skin. The cause of this may be the water-solubility of the steroid sulphates, or their lower affinity for the binding proteins. In adrenogenita

**Table X**

*Correlation between $C_{19}$-steroid sulphate contents in abdominal skin tissue and in serum of healthy women and healthy men and hirsute women with adrenogenital syndrome*

$$\text{Ratio} = \frac{\text{steroid conc. (nmol/kg) in abdominal skin tissue}}{\text{steroid conc. (nmol/l) in serum}}$$

| | | And-S | DHA-S | $\Delta^5$-diol-S | Test-S |
|---|---|---|---|---|---|
| Healthy women | mean | 0.08 | 0.01 | 0.12 | 1.12 |
| (28—46 years) | limits | 0.04—0.20 | 0.006—0.03 | 0.06—0.22 | 0.94—3.44 |
| n=7 | | | | | |
| Healthy men | mean | 0.10 | 0.21 | 0.07 | 1.71 |
| (28—47 years) | limits | 0.04—0.40 | 0.14—0.65 | 0.03—0.25 | 1.04—2.56 |
| n=5 | | | | | |
| R. A. 18 years | | 0.19 | 0.18 | 0.10 | 0.64 |
| O. S. 34 years | | 0.10 | 0.25 | 0.04 | 1.98 |

syndrome only DHA-S showed an accumulation relative to the level for normal women in both cases.

Few data have been found in the literature concerning the concentrations of steroids in skin tissue [18]. Deslypere et al. [2, 3] determined the concentrations of three androgenic steroids (Test., DHT and 5α-A-diol) in skin tissue from men. The evaluation of their results is hampered by the circumstance that they obtained their skin samples from corpses; the metabolism occurring up to the time of examination of the samples may have caused appreciable changes in the ratios of the individual steroids. Nevertheless, they demonstrated that the scrotal skin contained the most androgen; the concentration was lower in the pubic skin, and even lower in skin from the thigh. Thus, comparisons may be made only between steroid levels in skin tissue from the same area of the body. In every case we performed steroid analyses on skin tissue from the abdominal region, and thus the steroid concentrations found reflect the in vivo hormonal state of the abdominal skin.

An ever increasing number of data now support the assumption that the androgen-sensitivities of the various tissues can be characterized by the numbers of androgen-binding sites (receptors), the latter varying in parallel with the concentration of the androgens. Accordingly, the androgen accumulation (hyperandrogenism) found in the abdominal skin of the adrenogenital syndrome patients may be explained by the accumulation of certain binding proteins in the pathologically hairy skin. However, the proof of this must be the task of following investigations.

**Acknowledgements**

The authors wish to thank Professor F. A. László and Dr. J. Julesz for the evaluation of clinical data. They are also much indebted to Professor W. Klyne and Professor D. N. Kirk (London) for reference steroids.

REFERENCES

1. Bouchard, P., Kutten, F., Mowszowicz, I., Schaison, G., Raux-Eurin, M., Mauvais-Jarvis, P.: Congenital adrenal hyperplasia due to partial 21-hydroxylase deficiency, A study of five cases. Acta Endocrinol. **96,** 107—111 (1981)
2. Deslypere, J.-P., Sayed, A., Verdonck, L., Vermeulen, A.: Androgen contractions in sexual and non-sexual skin as well as in striated muscle in man. J. Steroid Biochem. **13,** 1455—1458 (1980)
3. Deslypere, J.-P., Vermeulen, A.: Aging and tissue androgens. J. Clin. Endocrinol. Metab. **53,** 430—434 (1981)
4. Faredin, I., Kókai, K., Tóth, I., Fazekas, Á. G., Julesz, M.: The metabolism of [4-$^{14}$C]dehydroepiandrosterone by human skin in vitro. Acta med. Acad. Sci. hung. **27,** 95—102 (1970)
5. Faredin, I., Szarvas, F., Tóth, I., Dávid, M., Julesz, M.: Pregnanetriol studies in hirsutism and other endocrine diseases. Acta med. Acad. Sci. hung. **20,** 327—338 (1964)
6. Faredin, I., Tóth, I.: Steroid studies on florisil adsorbent column V. Simultaneous determination of pregnantriol and total-17-hydroxycorticosteroids in human urine. Acta med. Acad. Sci. hung. **20,** 317—326 (1964)

7. Faredin, I., Tóth, I.: Biotransformation of [4-$^{14}$C]4-androstene-3,17-dione by healthy human skin in vitro. Acta med. Acad. Sci. hung. **32,** 129—137 (1975)
8. Faredin, I., Tóth, I.: The metabolism of [4-$^{14}$C]5-androstene-3$\beta$,17$\beta$-diol by normal human skin in vitro. Acta med. Acad. Sci. hung. **32,** 139—152 (1975)
9. Faredin, I., Tóth, I.: A [4-$^{14}$C]testosterone in vitro metabolizmusa egészséges emberi bőrben. Kísérl. Orvostud. **27,** 159—166 (1975)
10. Faredin, I., Tóth, I.: Metabolism of androgenic steroids in human skin of patients with various endocrine disorders. In: László F. A.: Recent results in peptide hormone and androgenic steroid research. pp. 197—207 Akadémiai Kiadó, Budapest, 1979
11. Faredin, I., Tóth, I.: Investigation of free $C_{19}$-steroids and their sulphate esters in human skin (Abstract). pp. 101—102 Proc. 20$^{th}$ Hung. Ann. Meet. Biochem., 1980
12. Fehér, T.: Some newly recognized correlation of steroid hormone action, tissue level of hormones and their biosynthetic processes (Abstract). Acta Biochim. et Biophys. Acad. Sci. Hung. **17,** 42 (1982)
13. Gláz Edit: A mellékvesekéreg kevert, hypo- és hyperfunctiója. In: Gláz Edit: Klinikai endocrinologia. p. 606 Med. Kiadó, Budapest, 1981
14. Julesz, M., Faredin, I., Tóth, I.: Steroids in human skin. Akadémiai Kiadó, Budapest, 1971.
15. Loriaux, D. L., Ruder, H. J., Lipsett, M. B.: Plasma steroids in congenital adrenal hyperplasia. J. Clin. Endocrinol. Metab. **39,** 627—630 (1974)
16. Solomon, I. L., Schoen, E. J.: Blood testosterone values in patients with congenital virilizing adrenal hyperplasia. J. Clin. Endocrinol. Metab. **40,** 355—362 (1975)
17. Takayasu, S., Wakimoto, H., Itami, S., Sano, S.: Activity of testosterone 5$\alpha$-reductase in various tissues of human skin. J. Invest. Dermatol. **74,** 187—191 (1980)
18. Tóth, I., Julesz, J., Faredin, I., László, F. A.: Investigation of androgenic steroids in the plasma of healthy subjects and endocrine patients (Abstract). Proc. 20th Hung. Ann. Meet. Biochem., 1980, pp 99—100.
19. Tóth, I., Faredin, I.: Separation and identification of free and esterified 17-ketosteroids. Acta med. Acad. Sci. hung. **29,** 141—144 (1972)
20. Tóth, I., Faredin, I.: Összefüggés az emberi verejték steroid tartalma és a plasma steroidszintje között. Kísérl. Orvostud. **30,** 348—356 (1978)
21. Tóth, I., Faredin, I.: Szabad androgen steroidok és $C_{19}$-steroid sulphatok az emberi hónaljszőrzetben. Kísérl. Orvostud. **30,** 637—645 (1978)
22. Tóth, I., Faredin, I.: A testosteron, az 5$\alpha$-dihydrotestosteron, az 5-androsten-3$\beta$,17$\beta$-diol és a 4-androsten-3,17-dion szimultán meghatározása emberi serumból. Kísérl. Orvostud. **30,** 55—65 (1978)
23. Tóth, I., Faredin, I.: $C_{19}$-steroid-sulphatok az emberi verejtékben. Kísérl. Orvostud. **33,** 159—165 (1981)
24. Tóth, I., Faredin, I.: Concentrations of androgens and $C_{19}$-steroid sulphates in abdominal skin tissue of healthy subjects and women with hirsutism. Acta Biochim. et Biophys. Acad. Sci. Hung. **17,** 45 (1982)
25. Tóth, I., Faredin, I.: Steroids excreted by human skin I. $C_{19}$-steroids in axillary hair. Acta med. Acad. Sci. Hung. **40,** 139—145 (1983)
26. Voigt, W., Fernandez, E. P., Hsia, S. L.: Transformation of testosterone into 17$\beta$-hydroxy-5$\alpha$-androstan-3-one by microsomal preparations of human skin. J. biol. Chem. **245,** 5594—5599 (1970)

*Acta Medica Hungarica, 41 (1), pp. 19—34 (1984)*

# METABOLISM AND CONCENTRATION OF ANDROGENIC STEROIDS IN THE ABDOMINAL SKIN OF WOMEN WITH IDIOPATHIC HIRSUTISM

I. FAREDIN and I. TÓTH

ENDOCRINE UNIT AND RESEARCH LABORATORY, FIRST DEPARTMENT OF MEDICINE, UNIVERSITY MEDICAL SCHOOL, SZEGED, HUNGARY

Received: February 28, 1983

The in vitro metabolisms of [4-$^{14}$C]-labelled DHA, $\Delta^5$-diol, $\Delta^4$-dione and Test were studied in skin tissue excised from the hairy hypogastric region of three patients diagnosed as suffering from "idiopathic hirsutism". The concentrations of DHA, And, $\Delta^4$-dione, $\Delta^5$-diol, Test, DHT, DHA-S, And-S, $\Delta^5$-diol-S and Test-S were determined in other portions of the same skin tissue.

In the knowledge of the concentrations of the androgens and the $C_{19}$-steroid sulphates in the blood and in the skin tissue, and also of the metabolisms of the main androgen precursors and Test in the hairy abdominal skin, new diagnoses can be established within the group of idiopathic hirsutisms: "pure peripheral hirsutism" and "mixed peripheral hirsutism". In the former the hyperactivity of the enzymes of the skin tissue takes part in the emergence of the disease form, while the latter involves the joint participation of the hyperactivity of the enzymes of the skin tissue and the high level of $\Delta^4$-dione in the blood.

The picture of the metabolism in the hairy abdominal skin of the hirsute patients was dominated by Test formed in pathologically high amount from the precursors as a consequence of the hyperactivity of 17$\beta$-HSD. The formation of DHT and the activity of 5$\alpha$-R were of only secondary importance.

**Keywords:** idiopathic hirsutism, human skin, steroid metabolism

## Introduction

Hirsutism is a special disease of women, which finds no parallel in the animal world. A hirsute woman endures her disease throughout her life. Her feeling on inferiority causes her not to marry, or if she is already married her family life may undergo a crisis. Perhaps nowhere does the clinician encounter so much disappointment as in the diagnostics and therapy of hirsutism.

Send offprint requests to I. Faredin H-6701 Szeged P.O.B. 469, Hungary

The following abbreviations are used: 17-KS = 17-Ketosteroids; 17-OH-CS = 17-Hydroxycorticosteroids; P-triol = 5$\beta$-pregnane-3$\alpha$,17$\alpha$,20$\alpha$-triol; Test = 17$\beta$-hydroxy-4-androstene-3-one; DHT=17$\beta$-hydroxy-5$\alpha$-androstane-3-one; 5$\alpha$-ane-3$\alpha$,17$\beta$-diol=5$\alpha$-androstane-3$\alpha$,17$\beta$-diol; 5$\alpha$-ane-3$\beta$,17$\beta$-diol = 5$\alpha$-androstane-3$\beta$,17$\beta$-diol; $\Delta^5$-diol = 5-androstene-3$\beta$,17$\beta$-diol; $\Delta^4$-dione = 4-androstene-3,17-dione; 5$\alpha$-ane-dione = 5$\alpha$-androstane-3,17-dione; And = 3$\alpha$-hydroxy-5$\alpha$-androstane-17-one; Epiand = 3$\beta$-hydroxy-5$\alpha$-androstane-17-one; DHA = 3$\beta$-hydroxy-5-androstene-17-one; And-S = 3$\alpha$-sulphooxy-5$\alpha$-androstane-17-one; DHA-S = 3$\beta$-sulphooxy-5-androstene-17-one; $\Delta^5$-diol-S = 5-androstene-3$\beta$,17$\beta$-diol-3-sulphate; Test-S = 17$\beta$-sulphooxy-4-androstene-3-one.

Hirsutisms of unknown origin are referred to in the literature under the collective name "idiopathic hirsutism" (I. H. [24, 31, 36, 43]). About 40% of our own hirsutism cases involve I. H. This means that the available methodological diagnostic possibilities fail in almost every second case [25, 36].

The failures have convinced us that the previously employed methods can not lead us further in the approach to the problematics of hirsutism. Accordingly, we began to make a study of the connection between the skin and the hormonal activity of the endocrine system from the side of the periphery, i.e. the skin.

It is known from previous experiments that pubic and abdominal skin slices intensively metabolize radioactive dehydroepiandrosterone (DHA), 4-androstene-3,17-dione ($\Delta^4$-dione), testosterone (Test) and 5-androstene-$3\beta$,$17\beta$-diol ($\Delta^5$-diol) under in vitro conditions [7, 8, 10, 11, 14—16, 21]. These investigations demonstrated that human abdominal skin contains numerous enzymes which are of great importance from the aspect of the androgen metabolism. We have therefore carried out in vitro incubation studies with hairy abdominal skin slices excised from the suprapubic abdominal area of our I. H. patients, and with [4-$^{14}$C]-labelled DHA, $\Delta^5$-diol, $\Delta^4$-dione and Test, in order to acquire information on the activities of the enzymes metabolizing the androgens in the hairy abdominal skin of our patients.

In parallel with these experiments, we have examined the concentrations of the free androgens and the $C_{19}$-steroid sulphates in the excised hairy abdominal skin slices, to decide whether the hyperandrogenism assumed in the hairy abdominal skin of I. H. patients [1, 24, 25] can be confirmed.

The present publication gives an account of the results of these experiments.

## Chemicals, patients and methods

*Chemicals*

The solutions used in the examinations were prepared from p.a. grade chemicals dissolved in water distilled from glass.

The [4-$^{14}$C]-labelled radioactive steroids DHA (specific activity [S.A.] = 57.1 mCi/mmol), $\Delta^5$-diol (S.A. = 57.1 mCi/mmol), $\Delta^4$-dione (S.A. = 56.6 mCi/mmol) and Test (S.A. = 22.19 mCi/mmol); and the [7-$^3$H(N)]-DHA (S.A. = 24 Ci/mmol), [1$\alpha$,2$\alpha$(n)-$^3$H] DHT (S.A. = 60 Ci/mmol), [1,2-$^3$H(n)]$\Delta^4$-dione (S.A. = 45 Ci/mmol) and [1$\beta$,2$\beta$-$^3$H]Test (S.A. = 44.6 Ci/mmol) were products of the Radiochemical Centre (Amersham, England). The [7-$^3$H(N)]$\Delta^5$-diol (S.A. = 20 Ci/mmol), [1,2-$^3$H(N)]-And (S.A. = 40 Ci/mmol) and the ammonium salt of [7-$^3$H(N)]DHA-S (S.A. = 24 Ci/mmol) were purchased from New England Nuclear (Boston, U.S.A.).

Before use, the free radioactive steroids were purified on a 4 g $Al_2O_3$ column (E. Merck, Brockmann III/IV activity), and their purities were then checked by chromatography on an $Al_2O_3$-60 G thin layer (E. Merck Type E) in the TLC-G system (n-hexane—ethyl acetate—abs. ethanol—glacial acetic acid = 120 : 130 : 1 : 2 by volume [24].

The sulphate ester steroids (DHA-S, $\Delta^5$-diol-S) were purified on a 1.5 g florisil column (60/100 mesh, Floridin Co., Tallahassee, U.S.A.) before use [46], and their purities were checked by chromatography according to Sarfaty and Lipsett on a silica gel G (E. Merck) thin layer

in the TLC-I System (abs. ethanol—ethyl acetate—conc. $NH_4OH$ = 50 : 50 : 10 by volume) [24].

The non-radioactive authentic steroids were purchased from Ikapharm (Ramat-Gan, Israel). Their purification and the checks on their purities were performed as described above.

*Patients*

Two 16-year-old (M. I. and T. Á.) and one 17-year-old (K. E.) girls were examined in our Unit; they complained that they had observed an enhanced growth of hair all over the body, which required cosmetic treatment. Their pubic hair was found to be of male type, while an enhanced growth of hair could be seen on their faces, around their nipples, and on their lower limbs. Their blood pressures were normal. Clinical and gynaecological examination showed normal conditions. Their menstruation cycles were regular.

The listed laboratory results indicated that the hirsutism was idiopathic in all three patients (Table I—III).

**Table I**

*Urinary steroid excretion in women with idiopathic hirsutism (μmol/d)*

| Patients | Total 17-Ks* | Total 17-OH-CS** | P-triol*** |
|---|---|---|---|
| M. I. 16 years | 30.5 | 7.4 | 2.7 |
| | 33.3 | 9.7 | 1.2 |
| T. A. 16 years | 51.7 | 11.6 | 2.4 |
| | 39.2 | 10.5 | 1.8 |
| K. E. 17 years | 39.2 | 10.2 | 2.4 |
| | 40.9 | 11.0 | 2.7 |
| Normal women (16—50 years) | 17.4—52.0 | 5.0—16.6 | 1.2—7.7 |

* Total 17-Ketosteroids
** Total 17-Hydroxycorticosteroids
*** Pregnanetriol (5β-pregnane-3α,17α, 20α-triol)

**Table II**

*Serum androgen levels in women with idiopathic hirsutism (nmol/l)*

| Patients | Test | DHT | $\Delta^5$-diol | $\Delta^4$-dione |
|---|---|---|---|---|
| M. I. 16 years | 2.6 | 0.89 | 3.0 | 3.9 |
| T. A. 16 years | 2.5 | 0.48 | 3.3 | 13.3 |
| K. E. 17 years | 2.9 | 1.27 | 1.9 | 11.5 |
| Normal women (16—50 years) | | | | |
| mean: | 2.4 | 2.2 | 3.7 | 6.3 |
| range: | 0.87—3.29 | 0.34—3.79 | 2.10—5.61 | 2.97—9.36 |

**Table III**

*Serum $C_{19}$-steroid sulphate and cortisol levels in women with idiopathic hirsutism*

| Patients | And-S μmol/l | DHA-S μmol/l | $\Delta^5$-diol-S μmol/l | Test-S nmol/l | Cortisol nmol/l |
|---|---|---|---|---|---|
| M. I. 16 years | 3.18 | 7.87 | 0.66 | — | 370 |
| T. A. 16 years | — | 4.54 | — | — | 364 |
| K. E. 17 years | 0.89 | 7.52 | 0.19 | 9.93 | 419 |
| Normal women (16—50 years) | | | | | |
| mean: | 1.57 | 8.28 | 0.35 | 12.05 | 345 |
| range: | 0.67—3.40 | 3.04—11.89 | 0.19—0.65 | 4.07—14.95 | 220—550 |

*Skin samples*

Skin samples from healthy men and women were obtained during appendectomy under general anaesthesia. These individuals were not suffering from endocrine or other diseases.

With the consent of the three I. H. patients, skin was excised from the suprapubic area under general anaesthesia. In all three cases the skin samples were very hairy.

The skin samples were purified from antiseptic and from the accessory fat tissues. Experimental processing occurred within 20 minutes of surgery.

The abdominal skin samples used for examinations contained both dermis and epidermis.

*Incubation of skin tissues*

A portion of the cleaned abdominal skin samples was cut with scissors into 1—2 mm slices, and 0.5 g quantities were incubated separately with 0.5 μCi [4-$^{14}$C]DHA, [4-$^{14}$C]$\Delta^5$-diol, [4-$^{14}$C]$\Delta^4$-dione and [4-$^{14}$C]Test as described previously [10, 12, 13]. The metabolites formed were determined.

*Determination of concentrations of free androgens and $C_{19}$-steroid sulphates in abdominal skin tissue*

In another portion of the cleaned abdominal skin tissue, the concentrations of free androgens (DHA, And, $\Delta^4$-dione, $\Delta^5$-diol, Test and DHT) and $C_{19}$-steroid sulphates (DHA-S, And-S, $\Delta^5$-diol-3-S and Test-S) were determined as described previously [12, 13]. The concentrations of the individual steroids are given in units of nmol/kg wet skin tissue.

*Determination of concentrations of androgens and $C_{19}$-steroid sulphates in serum*

In the serum of blood samples taken at 8. a.m., Test, $\Delta^5$-diol, DHT and $\Delta^4$-dione were determined as described previously [47]. The results are expressed in units of nmol/l.

Of the $C_{19}$-steroid sulphates, the concentration of DHA-S in the serum was determined by direct radioimmunoassay (RIA). The results are expressed in units of μmol/l.

The concentrations of And-S, $\Delta^5$-diol-S and Test-S in the serum were determined with the P.B.A. procedure of Tóth [45]. The And-S and $\Delta^5$-diol-3-S concentrations are expressed in units of μmol/l, and the Test-S concentrations in units of nmol/l.

The serum cortisol level was determined with a modification of the extraction procedure of Mattingly and the fluorimetric procedure of Spencer—Peet [6, 30, 42].

The total 17-Ks, the total 17-OH-CS and the P-triol in the urine were determined as described previously [9].

## Results

### *Examinations of steroids in urine and blood*

For none of the three I. H. patients did the examination of the amounts of setroids excreted in the urine (Table I) reveal differences which could be correlated with the high degree of hirsutism.

In the 8 a.m. blood samples the serum total Test, DHT and $\Delta^5$-diol concentrations (Table II) were normal in all three patients. The $\Delta^4$-dione level was normal in patient M. T., but pathologically high in the other two cases.

The concentrations of the $C_{19}$-steroid sulphates (And-S, DHA-S, $\Delta^5$--diol-3-S and Test-S) and cortisol for the three I. H. patients (Table III) did not differ from the values for healthy women.

### *Metabolic examinations in abdominal skin*

The percentage conversions of the radioactive metabolites formed during the in vitro incubation of the radioactive steroid substrates and the hairy abdominal skin slices from the three I. H. patients were compared with the results obtained under similar conditions for healthy women (Tables IV-VII).

**Table IV**

*The in vitro metabolism of [4-$^{14}$C] dehydroepiandrosterone by human skin*

| [4-$^{14}$C]DHA | Healthy women (17—45 years) | Patients with idiopathic hirsutism | | | Healthy men (19—46 years) |
|---|---|---|---|---|---|
| | | M. I. 16 years | T. Á. 16 years | K. E. 17 years | |
| | conversion % | | | | |
| Test | 0.20 (0.08—0.33) n=3 | 1.43 | 0.14 | 0.49 | 0.48 (0.04—1.38) n=6 |
| $\Delta^5$-diol | 1.32 (1.03—1.90) n=3 | 3.51 | 2.52 | 2.35 | 1.85 (1.19—2.97) n=5 |
| $\Delta^4$-dione | 5.34 (0.85–13.00) n=7 | 15.20 | 1.45 | 13.67 | 6.00 (1.37—12.39) n=9 |
| 5α-ane-dione | 1.76 (0.85—2.22) n=3 | 2.50 | 0.40 | 1.35 | 1.86 (0.30—4.84) n=5 |
| And | 2.14 (0.81—3.70) n=4 | 6.13 | 0.30 | 1.92 | 2.53 (0.62—6.84) n=6 |

**Table V**

*The in vitro metabolism of [4-$^{14}$C] 5-androstene-3β,17β-diol by human skin*

| [4-$^{14}$C]$\Delta^5$-diol | Healthy women (22—46 years) n=3 | Patients with idiopathic hirsutism M. I. 16 years | T. Á. 16 years | K. E. 17 years | Healthy men (19—45 years) n=6 |
|---|---|---|---|---|---|
| | conversion % | | | | |
| Test | 18.73 (13.20—26.80) | 24.55 | 1.86 | 16.25 | 8.75 (4.06—15.13) |
| DHT | 0.67 (0.40—0.98) | 1.90 | 0.22 | 0.58 | 1.31 (0.30—2.72) |
| $\Delta^4$-dione | 5.23 (1.40—11.16) | 2.20 | 0.28 | 1.05 | 1.41 (0.45—4.08) |
| 5α-ane-dione | 0.53 (0.34—0.64) | 5.02 | 0.13 | 0.25 | 0.65 (0.30—1.86) |
| And | 0.57 (0.52—0.66) | 1.26 | 0.15 | 0.32 | 0.41 (0.28—0.54) |
| DHA | 4.92 (3.43—6.09) | 3.44 | 4.62 | 3.76 | 6.79 (3.40—20.98) |

**Table VI**

*The in vitro metabolism of [4-$^{14}$C]4-androstene-3,17-dione by human skin*

| [4-$^{14}$C]$\Delta^4$-dione | Healthy women (26—43 years) n=7 | Patients with idiopathic hirsutism M. I. 16 years | T. Á. 16 years | K. E. 17 years | Healthy men (19—37 years) n=5 |
|---|---|---|---|---|---|
| | conversion % | | | | |
| Test | 1.80 (1.33—2.28) | 2.65 | 3.63 | 2.30 | 1.96 (0.91—2.62) |
| DHT | 0.37 (0.10—0.56) | 1.85 | 0.69 | 0.43 | 0.54 (0.18—0.86) |
| 5α-ane-dione | 10.85 (4.20—16.14) | 42.80 | 17.26 | 12.00 | 12.93 (9.15—15.45) |
| And | 8.34 (2.57—11.42) | 17.00 | 16.62 | 13.33 | 10.82 (7.09—15.87) |
| Epiand | 6.01 (1.26—10.30) | 2.87 | 1.83 | 10.15 | 2.39 (0.98—4.85) |

*Androgens and $C_{19}$-steroid sulphates in abdominal skin tissue*

The results on the androgens and the $C_{19}$-steroid sulphates in the hairy abdominal skin from the three I. H. patients are given in Tables VIII-IX.

**Table VII**

*The in vitro metabolism of [4-$^{14}$C] testosterone by human skin*

| [4-$^{14}$C] Test | Healthy women (26—43 years) n=5 | Patients with idiopathic hirsutism | | | Healthy men (19—37 years) n=5 |
|---|---|---|---|---|---|
| | | M. I. 16 years | T. Á. 16 years | K. E. 17 years | |
| | conversion % | | | | |
| DHT | 5.61 (3.70—7.21) | 11.39 | 4.86 | 3.00 | 6.39 (3.84—9.33) |
| 5α-ane-diols | 3.45 (2.26—4.58) | 5.71 | 1.54 | 2.65 | 3.36 (1.20—8.51) |
| $\Delta^4$-dione | 3.52 (1.82—5.11) | 6.52 | 3.28 | 1.98 | 4.20 (3.43—6.30) |
| 5α-ane-dione | 0.81 (0.50—1.14) | 4.81 | 1.12 | 0.55 | 1.22 (0.50—1.78) |
| And | 0.83 (0.61—1.36) | 2.64 | 3.41 | 0.50 | 1.37 (0.57—2.69) |
| Epiand | 0.69 (0.33—1.59) | 0.28 | 0.24 | 0.40 | 0.71 (0.24—1.25) |

**Table VIII**

*Androgenic steroids in abdominal skin tissue from women with idiopathic hirsutism (nmol/kg wet weight)*

| Patients | DHA | And | $\Delta^4$-dione | $\Delta^5$-diol | Test | DHT |
|---|---|---|---|---|---|---|
| Patients with idiopathic hirsutism | | | | | | |
| M. I. 16 years | 716 | 105 | 248 | 203 | 63 | 264 |
| T. Á. 16 years | 66 | 181 | 132 | 70 | 51 | 48 |
| K. E. 17 years | 116 | 163 | 54 | 58 | 326 | 7 |
| Healthy women | | | | | | |
| mean | 79 | 55 | 36 | 30 | 17 | 9 |
| ±S.E.M. | ±29 | ±19 | ±8 | ±14 | ±6 | ±4 |
| (28—46 years) n=7 | | | | | | |

*Quotients of androgen concentrations in abdominal skin tissue and serum*

Table X lists the quotients of the concentrations of the androgens in the skin tissue and those in the serum, while the corresponding data for the $C_{19}$-steroid sulphates are to be found in Table XI.

**Table IX**

*$C_{19}$-steroid sulphates in abdominal skin tissue from women with idiopathic hirsutism (nmol/kg wet weight)*

| Patients | And-S | DHA-S | $\Delta^5$-diol-S | Test-S |
|---|---|---|---|---|
| Patients with idiopathic hirsutism | | | | |
| M. I. 16 years | 4.018 | 33.631 | 4.987 | 14 |
| T. Á. 16 years | 780 | 398 | 57 | 11 |
| K. E. 17 years | 1.069 | 6.889 | 699 | 173 |
| Healthy women | | | | |
| mean: | 132 | 82 | 42 | 14 |
| ±S.E.M. | ±29 | ±27 | ±7 | ±3 |
| (28—46 years) | | | | |
| n=7 | | | | |

**Table X**

*Correlation between androgenic steroid contents in abdominal skin tissue and in serum of women with idiopathic hirsutism*

| Patients | $\Delta^4$-dione | $\Delta^5$-diol | Test | DHT |
|---|---|---|---|---|
| M. I. 16 years | 63.6 | 67.7 | 24.2 | 29.6 |
| T. Á. 16 years | 9.9 | 21.2 | 20.4 | 100 |
| K. E. 17 years | 4.7 | 30.5 | 112.4 | 5.4 |
| Healthy women | | | | |
| mean: | 5.7 | 8.1 | 7.1 | 4.1 |
| range: | 3.8—12.1 | 5.3—14.3 | 5.2—19.5 | 2.4—26.5 |
| (28—46 years) | | | | |
| n = 7 | | | | |

**Table XI**

*Correlation between $C_{19}$-steroid sulphate contents in abdominal skin tissue and in serum of women with idiopathic hirsutism (abdominal skin tissue nmol/kg/serum nmol/l)*

| Patients | And-S | DHA-S | $\Delta^5$-diol-S | Test-S |
|---|---|---|---|---|
| M. I. 16 years | 1.26 | 4.28 | 7.55 | — |
| T. Á. 16 years | — | 0.09 | — | — |
| K. E. 17 years | 1.20 | 0.92 | 3.68 | 17.52 |
| Healthy women | | | | |
| mean: | 0.08 | 0.01 | 0.12 | 1.12 |
| range: | 0.04—0.20 | 0.006—0.03 | 0.06—0.22 | 0.94—3.44 |

## Discussion

Human skin and the accessory appendages (sebaceous and apocrine sweat glands, hair follicles) are currently regarded as typical androgen target organs [34, 35]. In skin tissues originating from the various anatomic regions (scrotum, prepuce, pubes, abdomen, face, etc.), an intensive androgen metabolism occurs, to an extent depending on the type of the skin [8, 10, 11, 14, 15, 23, 28]. Test is formed from the weakly androgen-active precursors (DHA, $\Delta^5$-diol, $\Delta^4$-dione). DHT is synthetized from Test in the skin: this is metabolized ot 5α-androstane--3α,17β-diol and to 5α-androstane-3β,17β-diol [28]. The enzymes $\Delta^5$-3β--hydroxysteroid dehydrogenase ($\Delta^5$-3β-HSD), 3α-HSD, 3β-HSD, 17β-HSD and $\Delta^4$-5α-reductase (5α-R) play important roles in these biochemical processes.

Relatively few authors have studied the metabolisms of the androgen precursors and Test in skin tissues from various anatomic regions in women with I. H. The findings are not clear-cut either. Thomas and Oake [44] observed an intensive metabolism of DHA in the in vitro incubates of skin tissues from the area of the linea alba in women with I. H. Particularly the formation of Test and the weakly androgen-active 17-Ks was enhanced in comparison with the values for healthy women. No difference in utilization of Test was observed between similar skin tissue from I. H. and healthy women. In contrast, Kuttenn et al. [28] and Mauvais-Jarvis et al. [32] demonstrated intensive DHT, 5α-ane 3α,17β-diol and 5α-ane 3β,17β-diol formation in the pubic skin of I. H. patients in the course of metabolism of Test.

Our investigations reveal that the hairy abdominal skin of the three I. H. patients metabolized the radioactive precursors (DHA, $\Delta^5$-diol, $\Delta^4$-dione) in different ways. A significant difference was also observed in the metabolism of radioactive Test (Table XII).

Although the blood androgen levels were normal, the hairy abdominal skin of patient M. I. formed Test, DHT and androgenic 17-Ks with pathologically high percentage conversions from DHA and $\Delta^4$-dione precursors. In the metabolism of [4-$^{14}$C] Test too, the syntheses of DHT, the 5α-ane-diols and the androgenic 17-Ks were pathologically enhanced: only the formation of Epiand. was diminished. These results indicate that the activities of 17β-HSD, 5α-R, $\Delta^5$-3β-HSD and 3α-HSD were enhanced in the hairy abdominal skin of the patient, while the activity of 3β-HSD was decreased. On the basis of our examinations, the hirsutism is regarded as the most severe in this case. Some support for this assumption is provided by the fact that the mother of this patient was also shown to suffer from hirsutism [29].

The hairy abdominal skin of patient T. Á. formed Test, DHT and the androgenic 17-Ks with pathologically high percentage conversions only from the precursor $\Delta^4$-dione. From the precursors DHA, $\Delta^5$-diol and Test, the androgenic metabolites were formed in normal or decreased amounts. These results

**Table XII**

*Activities of androgen precursor and testosterone metabolysing enzymes in abdominal hairy skin samples of women with idiopathic hirsutism*

| Metabolites \ Substrates | DHA | | | $\Delta^5$-diol | | | $\Delta^4$-dione | | | Test | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | M. I. 16 years | T. Á. 16 years | K. E. 17 years | M. I. 16 years | T. Á. 16 years | K. E. 17 years | M. I. 16 years | T. Á. 16 years | K. E. 17 years | M. I. 16 years | T. Á. 16 years | K. E. 17 years |
| Test (17$\beta$-HSD) | ↑ | N | ↑ | N | ↓ | N | ↑ | ↑ | ↑ | — | — | — |
| DHT (5$\alpha$-R) | — | — | — | ↑ | ↓ | N | ↑ | ↑ | N | ↑ | N | ↓ |
| $\Delta^5$-diol (17$\beta$-HSD) | ↑ | ↑ | ↑ | — | — | — | — | — | — | — | — | — |
| 5$\alpha$-ane-diols (3$\alpha$,-3$\beta$-HSD) | — | — | — | — | — | — | — | — | — | ↑ | ↓ | N |
| $\Delta^4$-dione ($\Delta^5$-3$\beta$-HSD) | ↑ | N | ↑ | N | ↓ | ↓ | — | — | — | ↑ | N | N |
| 5$\alpha$-ane-dione (5$\alpha$-R) | ↑ | ↓ | N | ↑ | ↓ | ↓ | ↑ | ↑ | N | ↑ | N | N |
| And (3$\alpha$-HSD) | ↑ | ↓ | N | ↑ | ↓ | ↓ | ↑ | ↑ | ↑ | ↑ | ↑ | ↓ |
| Epiand (3$\beta$-HSD) | — | — | — | — | — | — | N | N | N | ↓ | ↓ | N |
| DHA (17$\beta$-HSD) | — | — | — | N | N | N | — | — | — | — | — | — |

N = normal conversion
↑ = increased conversion
↓ = diminished conversion

HSD = hydroxysteroid dehydrogenase
R = reductase
y = years

require consideration, if it is borne in mind that the blood $\Delta^4$-dione level was pathologically high in this patient, while at the same time the activities of the enzymes 17$\beta$-HSD, 5$\alpha$-R and 3$\alpha$-HSD catalyzing the metabolism of this precursor were enhanced in the hairy abdominal skin.

The metabolisms of the precursors DHA and $\Delta^4$-dione were the most significant in the abdominal skin of patient K. E.: the metabolisms of $\Delta^5$-diol and Test did not play an appreciable role in the formation of the androgens. Besides the pathologically high blood level of $\Delta^4$-dione, the hyperactivities of 17$\beta$-HSD, $\Delta^5$-3$\beta$-HSD and 3$\alpha$-HSD could be detected in the hairy abdominal skin tissue.

Our investigations indicated that the deciding role in the hairy abdominal skin of the three I. H. patients is played by the hyperactivity of 17$\beta$-HSD, which catalyzes the conversion of $\Delta^4$-dione to Test. In this respect, the metabolism of $\Delta^4$-dione in the skin is of primary importance. The hyperactivity of 5$\alpha$-R, which synthetizes DHT from Test, is only of secondary importance. The hyperactivity of $\Delta^5$-3$\beta$-HSD plays a role in the metabolism of the precursor (DHA).

The hyperactivities of the enzymes in the hairy abdominal skin of the three I. H. patients suggested the possibility that the androgenic steroids are

formed locally in pathologically high amounts from various precursors reaching the skin from the blood stream.

The literature contains very few data on the steroid concentrations in skin tissue. Deslypere et al. [3] examined the concentrations of Test, DHT and 5α-ane-3α,17β-diol in the prostate gland, sexual skin (scrotum and pubes), non-sexual skin (thigh) and striated musculature (m. gastrocnemius) in corpses, within 24h of death. Their results showed that the androgen concentrations of the prostate gland and the sexual skin were much higher than those of the non-sexual skin and musculature. Deslypere and Vermeulen [4] studied the concentrations of Test, DHT and 5α-ane-3α,17β-diol in the same tissues post mortem as a function of age between 20 and 82 years. As the age increased, the concentrations of the three androgens decreased only in the pubic skin.

In these investigations the Test and DHT concentrations of the sexual and of the non-sexual skin were much lower than we found in the abdominal skin of healthy and of I. H. women. The explanation of this may be the difference between the two methods and the different manners of collecting the skin samples. Further experimentation should be carried out to clarify these points.

From the study of the free androgens it may be stated that all of the examined androgens were present in the hairy abdominal skin of patient M. I. in considerably higher concentrations than in healthy women. With the exception of DHA in patient T. Á. and DHT in patient K. E., all of the androgenic steroids were to be found in pathologically high amounts in the hairy abdominal skin of the other two cases. The extremely high Test concentration and the normal DHT concentration are striking.

Of the $C_{19}$-steroid sulphates, the concentrations of And-S, DHA-S and $\Delta^5$-diol-S were pathologically high in the hairy abdominal skin of all three I. H. patients. The concentration of Test-S was pathologically elevated in patient K. E., but normal in the other two patients.

The results demonstrate that most of the free androgens and $C_{19}$-steroid sulphates were present in much higher concentrations in the abdominal skin of the I. H. patients than in that of healthy women. Thus, the hyperandrogenism assumed on the basis of the in vitro incubation experiments was confirmed by androgen analysis of the abdominal skin tissues.

The extent of accumulation of the androgens and the $C_{19}$-steroid sulphates in the hairy abdominal skin of the I. H. patients can be expressed more illustratively by the quotients of their concentrations in the skin and in the serum.

All of the free androgens were accumulated considerably in the abdominal skin of patient M. I. In the abdominal skin of T. Á., the $\Delta^5$-diol, Test and DHT were accumulated to a pathological extent, while in patient K. E. the concentrations of $\Delta^5$-diol and Test were pathologically high, whereas that of DHT was normal. The $C_{19}$-steroid sulphates were accumulated considerably in the hairy abdominal skin of all three I. H. patients.

The quotients of the steroid concentrations in the abdominal skin tissue and in the serum confirmed that there were considerable accumulations of Test and $\Delta^5$-diol in the hairy abdominal skin of the three I. H. patients. The DHT concentration was pathologically high in patients M. I. and T. Á., while that of $\Delta^4$-dione was normal in patients T. Á. and K. E., in spite of the fact that the $\Delta^4$-dione level was pathologically high in the serum of these two patients.

It is known that human skin is unable to synthetize androgens from cholesterol or pregnenolone. Thus, the androgenic steroid content of human skin arises from Test, DHT and the weakly androgen-active precursors reaching the skin tissues from the blood stream. There is an intensive androgen metabolism in human skin, however, and the androgen precursors (DHA, $\Delta^5$-diol, $\Delta^4$-dione, etc.) are metabolized in various directions, depending on the activities of the enzyme systems of the skin, e.g. towards the strongly androgenic Test and DHT [8, 10, 11] and also towards sulpho-conjugation (DHA-S, $\Delta^5$-diol-S) [10, 15]. Therefore, the prevailing composition and quantity of the androgens in the skin tissue are determined jointly by the androgenic steroids formed locally in the skin or reaching the skin from the circulating blood.

On entering the blood stream, the bulk of the steroid hormones produced by the endocrine glands bind to protein (SHBG, transcortin, albumin), while the remainder circulates in the free state. The free hormones are of particular importance in this steroid hormone transport. If, for instance, the testosterone binding globulin (TeBG) level in the blood decreases, the free Test level in the blood rises: on passing into the skin and the hair follicles, it causes hirsutism [2, 43]. "Low TeBG level hirsutism" has thus been separated from the group of I. H. [2, 48].

The hirsutism is attributed to the enhanced sensitivity of the hair follicles if the level of total androgens circulating in the blood stream and the TeBG level are normal. In such cases, either the concentration of androgen receptors increases around the hair follicles, or the peripheral conversion increases in the skin, and pathologically high amounts of Test and DHT are formed from the precursors. Such hirsutism is regarded ad "pure peripheral hirsutism" [2].

Little is known of the state of the steroids in the tissues. An increasing number of data support the hypothesis that the androgen sensitivity of the tissues may be characterized by the number of binding sites of the androgen receptors. The latter varies in parallel with the concentration of the androgens [27]. Not only the androgens, but also the $C_{19}$-steroid sulphates exhibited high concentrations and accumulations in the hairy abdominal skin of our I. H. patients: this phenomenon can not be explained by the androgen receptors alone [34, 35]. It is possible that there is a change in the mechanism of elimination of the $C_{19}$-steroid sulphates formed in the abdominal skin or reaching it from the blood stream, but it is also possible that in the hairy abdominal skin

of I. H. women there is an accumulation of protein causing binding of the steroid sulphates examined [20, 26]. The importance of these steroid sulphates and their role, if any, in the development of hirsutism have not yet been elucidated.

Consideration of the above results leads to the following diagnoses for our three I. H. patients:

Patient M. I.: "pure peripheral hirsutism". This is supported by the following data:

1. The concentrations of androgens and $C_{19}$-steroid sulphates in the peripheral blood are normal. The cortisol and DHA-S levels are normal.

2. The metabolisms of Test and two precursors (DHA and $\Delta^4$-dione) in the hairy abdominal skin are enhanced. It follows that the enzymes 17$\beta$-HSD, 5$\alpha$-R, $\Delta^5$-3$\beta$-HSD and 3$\alpha$-HSD in the skin are hyperactive.

3. The concentrations and accumulations of the androgens in the hairy abdominal skin are pathologically high.

4. With the exception of Test-S, the concentrations of the $C_{19}$-steroid sulphates in the hairy abdominal skin are pathologically elevated. The accumulations of these steroids in the skin are similarly pathologically high.

Patient T. Á.: "mixed peripheral hirsutism". This is confirmed by the following results:

1. In the peripheral blood the Test, DHT and $\Delta^5$-diol levels are normal, while the concentration of $\Delta^4$-dione is pathologically high. The cortisol and DHA-S levels are normal.

2. The picture of the metabolism in the hairy abdominal skin is dominated by the metabolism of $\Delta^4$-dione. The activities of 17$\beta$-HSD, 5$\alpha$-R and 3$\alpha$-HSD in the skin are enhanced. The formation of DHT from Test is normal.

3. With the exception of DHA, the concentrations of the androgens in the hairy abdominal skin are pathologically high. With the exception of Test-S, the quantities of the $C_{19}$-steroid sulphates too are high.

4. With the exception of $\Delta^4$-dione, the accumulations of the androgens in the hairy abdominal skin are pathologically high. The accumulation of DHA-S is also enhanced.

Patient K. E.: "mixed peripheral hirsutism". This is supported by the following data:

1. In the peripheral blood, the levels of Test, DHT and $\Delta^5$-diol are normal, while the $\Delta^4$-dione concentration is pathologically high. The levels of cortisol, DHS and the $C_{19}$-steroid sulphates are normal.

2. The metabolisms of DHA and $\Delta^4$-dione are the most marked in the hairy abdominal skin. The metabolisms of $\Delta^5$-diol and Test do not play a significant role in the formation of the skin androgens. It follows that the activities of 17$\beta$-HSD, $\Delta^5$-3$\beta$-HSD and 3$\alpha$-HSD are enhanced. The formation of DHT from Test is decreased.

3. With the exception of DHT, the concentrations of the androgens and the $C_{19}$-steroid sulphates in the hairy abdominal skin are pathologically high.

4. The accumulation of Test in the hairy abdominal skin is extremely high, and the accumulation of $\Delta^5$-diol too is pathologically enhanced. The $\Delta^4$-dione and DHT levels are normal.

5. The accumulations of all the $C_{19}$-steroid sulphates in the hairy abdominal skin are pathologically high.

To evaluate our results, it may be stated that study of the levels of the androgens and the $C_{19}$-steroid sulphates in the blood and in the skin tissue, together with study of the metabolisms of the androgens in the skin tissue, permits the distinction of certain forms of I. H.: "pure peripheral hirsutism" and "mixed peripheral hirsutism". In the former, a role is played only by the hyperactivity of the enzyme systems of the skin tissue: in the latter, parts are played jointly by the hyperactivity of the enzyme systems of the skin tissue and by the high blood level of $\Delta^4$-dione.

The development and growth of sexual hair (moustache, beard, chest hair, etc.) is mainly a consequence of the effects of genetic and hormonal factors. Of the hormonal factors, special significance is attributed to the androgens [5, 22, 23]. An intensive androgen metabolism is known to take place in the hair follicles [18, 19, 39—41]. It was previously not known what roles are played by Test, DHT or other androgens in the development of the hair follicles and in the growth of the hair. Farthing et al. [17] examined the connection between the growth of hair on the face of men and the concentrations of Test and DHT in the plasma. Their investigations appear to confirm that Test and DHT play independent roles in the growth of face hair on men. Test influences the development of the hair follicles and the thickness of the hair, while DHT influences the growth of the length of the hair. Their results are supported by their studies on coeliac patients: the blood Test level was enhanced, the blood DHT level was decreased, the thickness of the facial hair was significantly higher, and the growth in length of the facial hair was considerably diminished. These results are in accordance with those of Randall et al. [37, 38], who demonstrated in experiments on rats that the mechanisms of action of the androgens in the skin differ from those observed in the prostate gland. The role of DHT is not so important in the skin as in the prostate gland, and Test itself is responsible for the androgen effect in the skin.

The above studies support what we observed in our hirsutism patients. The picture of the metabolism was dominated by the pathologically high quantity of Test formed from the precursors, as a consequence of the hyperactivity of 17$\beta$-HSD, this was confirmed by the pathologically high concentration of Test in the hairy abdominal skin. The formation of DHT and the activity of the enzyme 5$\alpha$-R are only of secondary importance in the hirsutism cases we examined. Further investigations are required to recognize the binding proteins

responsible for the accumulation of the androgens and $C_{19}$-steroid sulphates in the hairy skin.

## Acknowledgement

The authors wish to thank Professor F. A. László and Dr. J. Julesz for the evaluation of clinical data. They are also much indebted to Professor W. Klyne and Professor D. N. Kirk (London) for reference steroids.

## REFERENCES

1. Burton, J. L., Johnson, C., Libman, L., Shuster, S.: Skin virilism in women with hirsutism. J. Endocr. **53,** 349—354 (1972)
2. Dray, F., Sebaoun, J., Delzant, G., Ledru, M. J., Mowszowich, J.: Activité de liaison de la testosterone dans le sérum des femmes présentant un virilisme pilaire idiopathique. Rev. Franç et Clin. Biol. **13,** 622—624 (1968)
3. Deslypere, J. P., Sayed, A., Verdonck, L., Vermuelen, A.: Androgen concentrations in sexual and non-sexual skin as well as in striated muscle in man. J. Steroid Biochem. **13,** 1455—1458 (1980)
4. Deslypere, J. P., Vermeulen, A.: Aging and Tissue Androgens. J. clin. Endocr. **53,** 430—434 (1981)
5. Ebling, F. J.: Hair J. Invest. Derm. **67,** 98—105 (1976)
6. Faredin, I., Czakó, L., Tóth, I.: A cortisol fluorimetriás meghatározása emberi serumból. Kísérl. Orvostud. **26,** 380—387 (1974)
7. Faredin, I., Fazekas, Á. G., Kókai, K., Tóth, I., Julesz, M.: The in vitro metabolism of [4-$^{14}$C] dehidroepiandrosterone by human male pubic skin. Eur. J. Steroids **2,** 223—242 (1967)
8. Faredin, I., Kókai, K., Tóth, I., Fazekas, Á. G.: The metabolism of [4-$^{14}$C] dehydroepiandrosterone by human skin in vitro I. Transformation in vitro of [4-$^{14}$C] dehydroepiandrosterone into 4-androsterone-3,17-dione, testosterone and androsterone by normal human male and female skin slices. Acta med. Acad. Sci. hung. **27,** 95—102 (1970)
9. Faredin, I., Tóth, I.: Steroid studies on florisil adsorbent column. V. Simultaneous determination of pregnanetriol and total-17-hydroxycorticosteroids in human urine. Acta med. Acad. Sci. hung. **20,** 317—326 (1964)
10. Faredin, I., Tóth, I.: The metabolism of [4-$^{14}$C]5-androstene-3$\beta$,17$\beta$-diol by normal human skin in vitro. Acta med. Acad. Sci. hung **32,** 139—152 (1975)
11. Faredin, I., Tóth, I.: Biotransformation of [4-$^{14}$C]4-androstene-3,17-dione by healthy human skin in vitro. Acta med. Acad. Sci. hung. **32,** 129—137 (1975)
12. Faredin, I., Tóth, I.: Metabolism of androgen steroids in human skin in patients with various endocrine disorders. pp. 197—207 In: Recent results in peptide hormone and androgenic steroid research. (Ed. by László F. A.) Akadémiai Kiadó, Budapest, 1979
13. Faredin, I., Tóth, I.: Investigation of free $C_{19}$-steroids and their sulphate esters in human skin. pp. 101—102. Proc. 20th Hung. Ann. Meet. Biochem. Siófok, 1980
14. Faredin, I., Tóth, I., Fazekas, Á. G., Kókai, K., Julesz, M.: The in vitro metabolism of [4-$^{14}$C]4-androstene-3,17-dione by normal and hirsute female skin. Int. J. Derm. **9,** 147—152 (1970)
15. Faredin, I., Tóth, I., Julesz, M.: Metabolism of [4-$^{14}$C] dehydroepiandrosterone by human skin in vitro II. In vitro formation of water-soluble dehydroepiandrosterone sulphate and androst-5-ene-3$\beta$,17$\beta$-diol-3-sulphate in human abdominal skin. Acta med. Akad. Sci. hung. **30,** 91—99 (1973)
16. Faredin. I., Webb, J. L., Julesz, M.: The in vitro metabolism of dehydroepiandrosterone in human skin. Acta med. Acad. Sci. hung. **23,** 169—180 (1967)
17. Farthing, U. J. G., Mattei, A. U., Edwards, C. R. W., Dawson, A. U.: Relationship between plasma testosterone and dihydrotestosterone concentrations and male facial hair growth. Brit. J. Derm. **107,** 559—564 (1982)
18. Fazekas, Á. G., Lanthier, A.: Metabolism of androgens by isolated human beard hair follicles. Steroids **18,** 367—379 (1971)
19. Fazekas, Á. G., Sándor, T.: Metabolism of androgens by isolated human hair follicles. J. Steroid Biochem. **3,** 485—491 (1972)
20. Fehér, T.: Some newly recognized correlation of steroid hormone action, tissue level of hormones and their biosynthetic processes. Acta Biochim. et Biophys. Acad. Sci. hung. **17,** 42 (1982)

21. Gomez, E. C., Hsia, S. L.: In vitro metabolism of testosterone-4-$^{14}$C and $\Delta^4$-androstene-3,17-dione-4-$^{14}$C in human skin Biochemistry **7,** 24—32 (1968)
22. Hamilton, J. B.: Age, sex and genetic factors in the regulation of hair growth in man: a comparisons. p. 399 In: The Biology of Hair Growth (Ed. by W. Montagna and R. A. Ellis), Akademic Press, 1958
23. Hay, J. B., Hodgins, M. B.: Metabolism of androgens in vitro by human facial and axillary skin. J. Endocr. **59,** 475—486 (1973)
24. Julesz, J., Faredin, I., Tóth, I.: Steroids in human skin. Akadémiai Kiadó, Budapest, 1971
25. Julesz, M., Faredin, I., Tóth, I., Dávid, M. A., Kovács, K.: Studies of the urinary steroids in hirsutism and virilism. Acta med. Acad. Sci. hung. **19,** 311—324 (1963)
26. Katzenellenbogen, B. S.: Dynamics of steroid hormone receptor action. Ann. Rev. Physiol. **42,** 17—35 (1980)
27. Keenan, B. S., Meyer, W. J., Hadjian, A. J., Migeon, C. J.: Androgen receptor in human skin fibroblasts. Characterization of a specific 17$\beta$-hydroxy-5$\alpha$-androstan-3-one-protein complex in cell sonicates and nuclei. Steroids **25,** 535—552 (1975)
28. Kuttenn, F., Mowszowicz, I., Schaison, G., Mauvais-Jarvis, P.: Androgene production and skin metabolism in hirsutism. J. Endocr. **75,** 83—91 (1977)
29. Lorenzo, E. M.: Familial study of hirsutism. J. clin. Endocr. **31,** 556—564 (1970)
30. Mattingly, D.: A simple fluorimetric method for the estimation of free 11-hydroxycorticoids in human plasma. J. clin. Path. **15,** 374—379 (1962)
31. Mauvais-Jarvis, P., Kutten, F., I., Mowszowicz: Hirsutism. pp. 74—81. Springer-Verlag, Berlin, 1981
32. Mauvais-Jarvis, P., Kutten, F., Mowszowicz, I.: Androgen production and skin metabolism in idiopathic hirsutism. In: Recent results in peptide hormone and androgenic steroid research. pp. 223—233 (Ed. by László F. A.) Akadémiai Kiadó Budapest, 1979
33. Moretti, G., Rampini, E., Rebora, A.: The hair cycle re-evaluated. Int. J. Derm. **15,** 277—283 (1976)
34. Mowszowicz, I., Kopp, F., Martin, P.-M.: Multiple steroid binding sites in human skin cytosol. Brit. J. Derm. **107,** Suppl. **23,** 60—61 (1982)
35. Mowszowicz, I., Wright, F., Giacomini, M., Riahi, M.: Androgen receptors in human skin cytosol: physiological and pathological variations. Brit. J. Derm. **107,** Suppl. 23, 35 | 39 (1982)
36. Parker, F.: Skin and hormones. In: Textbook of Endocrinology. Sixth Edition. pp. 1094—1096 (Ed. by R. H. Williams) W. B. Saunders Co., Philadelphia, 1981
37. Randall, V. A., Ebling, F. J.: Is the metabolism of testosterone to 5$\alpha$-dihydrotestosterone required for androgen action in the skin? Brit. J. Derm. **107,** Suppl. 23, 47—53 (1982)
38. Randall, W. A., Ebling, F. J., Hargreaves, G.: In vivo uptake and metabolism of testosterone by the skin and other tissues of the rat. J. Endocr. **93,** 253—266 (1982)
39. Schweikert, H. U., Wilson, J. D.: Regulation of human hair growth by steroid hormones. I. Testosterone metabolism in isolated hairs. J. clin. Endocr. **38,** 811—819 (1974 a).
40. Schweikert, H. U., Wilson, J. D.: Regulation of human hair growth by steroid hormones. II. Androstenedione metabolism in isolated hairs. J. clin. Endocr. **39,** 1012—1019 (1974 b).
41. Schweikert, H. U., Wilson, J. D.: Aromatisation of androstenedione by isolated human hairs. J. clin. Endocr. **40,** 413—417 (1975)
42. Spencer-Peet, J., Daly, J. R., Smith, V.: A simple method for improving the specificity of the fluorimetric determination of adrenal corticosteroids in human plasma. J. Endocr. **31,** 235—244 (1965)
43. Strauss, J. S.: Hormones and the pilosebaceous apparatus. In: Hair Research, Status and future aspects. pp 223—228 (Ed. by Orfanos, C. E., Montagna, W., Stüttgen, G.) Springer-Verlag, Berlin, Heidelberg, New York, 1981
44. Thomas, J. P., Oake, R.: Androgen metabolism in the skin of hirsute women. J. clin. Endocr. **38,** 19—22 (1974)
45. Tóth, I.: Az emberi bőr által kiválasztott androgen steroidok. 91—96 old. Thesis, 1978.
46. Tóth, I., Faredin, I.: Separation and identification of free and esterified 17-ketosteroids. Acta med. Acad. Sci. hung. **29,** 141—144 (1972)
47. Tóth, I., Faredin, I.: A testosteron, az 5$\alpha$-dihydrotestosteron, az 5-androsten-3$\beta$,17$\beta$-diol és a 4-androsten-3,17-dion szimultán meghatározása emberi szérumból. Kísérl. Orvostud. **30,** 55—65 (1978)
48. Vermeulen, A., Rubens, R.: Biochemical exploration of hirsutism. In: Recent results in peptide hormone and androgenic steroid research. pp. 215—221 (Ed. by László F. A.) Akadémiai Kiadó, Budapest, 1979

*Acta Medica Hungarica, 41 (1), pp. 35—41 (1984)*

# EFFECT OF STEROIDS ON ACTH RELEASE FROM CULTURED PITUITARY CELLS OF THE RAT

J. JULESZ, M. LIS[2], J. GATKOWSKA[2], I. MIZSEI and J. GENEST[2]

ENDOCRINE UNIT AND RESEARCH LABORATORY, FIRST DEPARTMENT OF MEDICINE, H-6701 SZEGED, P.O.B. 469, AND
[1]BIOMETRICAL CENTRE, UNIVERSITY MEDICAL SCHOOL, SZEGED, HUNGARY, AND
[2]CLINICAL RESEARCH INSTITUTE OF MONTREAL, MONTREAL, QUEBEC, CANADA

Received: February 28, 1983

Using a primary cell culture of rat anterior pituitary and corticotropin (ACTH) RIA, the effects of various steroids have been investigated. Dexamethasone inhibited the ACTH release stimulated by α-melanotropin and Pitressin (posterior pituitary extract), but did not influence the non-stimulated release. The effect of all the investigated steroids was significant; aldosterone exhibited a more marked inhibition than spironolactone. Simultaneous administration of aldosterone and spironolactone resulted predominantly in the manifestation of spironolactone action. A new effect of spironolactone has been demonstrated in vitro; the evaluation of its pharmacological importance requires studies in vivo.

**Keywords:** corticotropin, dexamethasone, aldosterone, spironolactone, α-melanotropin, Pitressin

## Introduction

Pitressin$^R$ (Parke-Davis) is a sterile, aqueous solution of vasopressin. The manufacturers have recently changed the method of preparation: the earlier purified extract of bovine posterior pituitaries, substantially free from oxytocin, has been replaced by synthetic arginine-8-vasopressin (AVP). We have published our experiences with the corticotropin-releasing (CRF) activities of both Pitressin$^R$ preparations [10]: the earlier Pitressin$^R$ (posterior pituitary extract) caused a more pronounced stimulation of ACTH release in vitro as compared to the effect of the new synthetic preparation.

Consequently, we presumed that the preparation from posterior pituitaries contained a substance or substances which enhanced the corticotropin-releasing activity of pure vasopressin. It was earlier reported that Pitressin$^R$ (posterior lobe extract) is contaminated with α-melanotropin (α-MSH) [24]. We have recently demonstrated the CRF-like activity of α-MSH and the synergism of the combination of α-MSH and AVP in vitro [15].

In further studies, we tested the inhibition of ACTH release stimulated by α-MSH and Pitressin$^R$ in primary cell cultures of the rat anterior pituitary [11]. Some of our results with various steroids as inhibitors are presented in this paper.

Send offprint requests to Dr. J. Julesz, H-6701 Szeged, P.O.B. 469.

## Materials and methods

Pitressin$^R$ (posterior pituitary extract, Parke—Davis) and synthetic α-MSH (MW 1665.0, Dr. N. Ling) were used as stimulants of ACTH release; the inhibitory actions of dexamethasone (MW 392.45, Sigma), aldosterone (MW 360.44, Merck) and spironolactone (MW 416.60, Sigma) were challenged. The cell culture method was a modification of the technique of Vale et al. [21]. Twenty Fisher 344/Lis male rats of 220—280 g body weight were decapitated, and the anterior pituitaries carefully separated from the intermediate-posterior lobes. The cells of the anterior lobes were cultured as described previously [16]. The experimental incubation period was 3 hours: steroids were added to the cell cultures at the start of the experiment, followed 1 h later by the stimulants, with subsequent incubation for a further 2 h. A similar schedule has been employed by others [1, 2, 18] because steroids exert their effect through de novo synthesis of ribonucleic acid, and consequently the onset of their action requires a longer time than that for peptide hormones [14]. After the experiment, the incubation medium was removed for ACTH radioimmunoassay (RIA); in certain cases, for determination of the internal ACTH content of the cells, the cell walls were lysed with a buffer containing paramethylsulphonyl fluoride, iodoacetamide and detergent, and by repeated freezing. ACTH measurement was performed in a 1 : 4 dilution of the medium and a 1 : 100 dilution of the lysed cells. The experimental medium and the lysing buffer did not interfere with the hormone determination.

The details of RIA for ACTH have been published elsewhere [6]. The assay was sensitive (1.08 pmol/l) and reproducible (coefficient of variation 11.3%). The antiserum used in the RIA was specific for the $NH_2$ terminus of ACTH, but it showed only a weak cross-reactivity with α-MSH (0.16% at 50% displacement of $^{125}$I-ACTH 1—39), and no cross-reactions with AVP or the investigated steroids.

The measured values should be considered as ACTH immunoreactivity. Control incubations in the absence of pituitary cells and at appropriate concentrations of the tested preparations were estimated simultaneously and the values of cross-reactivity subtracted when necessary. The latter never exceeded 20% of the immunoreactive material produced by the cells.

The steroids were dissolved in absolute ethanol and serial dilutions were made with the cell culturing medium, leading to final ethanol concentrations in the interval $4\times10^{-7}$ — — $4\times10^{-1}$ v/v%. Ethanol at a concentration of $4\times10^{-1}$ v/v%, was added also without steroid to the media above the untreated control cells and those treated only by stimulation. Ethanol at the said concentration did not affect the release of ACTH.

### *Statistical analyses*

The experiments were repeated 5 times and resulted in similar responses to the steroids. One representative experiment of each was chosen for presentation.

Mean of ACTH concentrations are given in pmol/l ± S.D. Data were analysed by Student's two-tailed *t* test, Dunnett's test, and one-way analysis of variance.

## Results

The effect of high doses ($10^{-5}$ g/ml) of α-MSH and dexamethasone on the pituitary cell culture medium is shown in Figure 1. Administration of α-MSH alone resulted in a significantly increased ACTH release. Dexamethasone inhibited the ACTH release stimulated by α-MSH, but did not influence the ACTH concentration in the medium of non-stimulated cells. The internal ACTH content of the cells was not affected by either α-MSH or dexamethasone.

We also tested the effect of various concentrations of dexamethasone on ACTH release stimulated by 1 U/ml of Pitressin$^R$ (Table I). For doses of $10^{-5}$—$10^{-9}$ g/ml, the inhibitory action of dexamethasone was significant and dose-dependent.

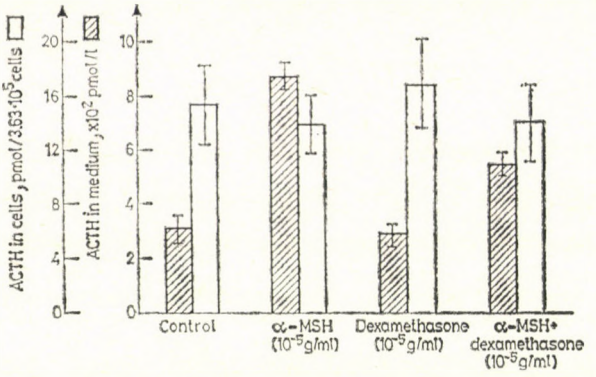



*Fig. 1.* Inhibitory effect of dexamethasone on α-MSH-stimulated ACTH release. ACTH in the medium and in the cells of rat anterior pituitaries mean ± S.D. (n = 4). The stimulatory effect of α-MSH on ACTH release was significant by Dunnett's test as compared to the release from untreated control cells. Dexamethasone did not affect the non-stimulated ACTH release (Dunnett), but significantly suppressed the stimulatory action of α-MSH (Student's two-tailed *t* test, $P < 0.001$). The internal ACTH content of the cells was not affected by any of the treatments (Dunnett)

Various concentrations of aldosterone, spironolactone and spironolactone + aldosterone were challenged in simultaneously performed experiments (Table II a, b and c). Pitressin$^R$ in a concentration of 1 U/ml was used as stimulant. Treatment of the cells with $10^{-5}$—$10^{-10}$ g/ml doses of aldosterone or spironolactone induced a significant and dose-related decrease in stimulated ACTH. Inhibition was also observed with $10^{-5}$—$10^{-8}$ g/ml concentrations of their combination (smaller doses of this combination were not investigated).

**Table I**

*Inhibitory effect of dexamethasone on ACTH release stimulated by 1 U/ml of Pitressin* n $^{R_1}$ *(posterior pituitary extract)*
ACTH concentration in medium[2] (pmol/l)

| Dexamethasone, g/ml | 0 | $10^{-5}$ | $10^{-6}$ | $10^{-7}$ | $10^{-8}$ | $10^{-9}$ | $10^{-10}$ | $10^{-11}$ |
|---|---|---|---|---|---|---|---|---|
| n | 7 | 8 | 4 | 4 | 4 | 4 | 4 | 4 |
| mean | 338.36 | 171.16 | 149.38 | 231.44 | 239.36 | 254.32 | 334.40 | 370.04 |
| ±S.D. | 34.91 | 42.04 | 40.26 | 44.66 | 31.55 | 43.03 | 52.29 | 45.78 |
| level of significance with Dunnett's test related to cells not treated with dexamethasone (0) | | 99% | 99% | 99% | 99% | 99% | NS | NS |

[1] ACTH release was stimulated by 1 U/ml of Pitressin$^R$ in each column of the Table.
[2] Mean of 12 control incubations was subtracted from the values obtained with treated cells.

## Table II

*Inhibitory effect of aldosterone and spironolactone on ACTH release stimulated by 1 U/ml of Pitressin $^{R}$[1] (posterior pituitary extract)*
ACTH concentration in medium[2] (pmol/l)

*a* Effect of aldosterone

| Aldosterone, g/ml | 0 | $10^{-5}$ | $10^{-6}$ | $10^{-7}$ | $10^{-8}$ | $10^{-9}$ | $10^{-10}$ | $10^{-11}$ |
|---|---|---|---|---|---|---|---|---|
| n | 7 | 8 | 4 | 4 | 4 | 4 | 4 | 4 |
| mean | 338.36 | 60.72 | 74.58 | 106.92 | 154.88 | 178.42 | 263.56 | 322.08 |
| ±S.D. | 34.91 | 35.82 | 35.22 | 8.58 | 28.12 | 44.70 | 43.74 | 44.18 |
| level of significance with Dunnett's test related to cells not treated with steroids (0) | | 99% | 99% | 99% | 99% | 99% | 99% | NS |

*b* Effect of spironolac tone

| Spironolactone, g/ml | 0 | $10^{-5}$ | $10^{-6}$ | $10^{-7}$ | $10^{-8}$ | $10^{-9}$ | $10^{-10}$ | $10^{-11}$ |
|---|---|---|---|---|---|---|---|---|
| n | 7 | 4 | 6 | 4 | 4 | 7 | 4 | 4 |
| mean | 338.36 | 163.68 | 170.94 | 198.44 | 213.18 | 201.52 | 251.46 | 338.80 |
| ±S.D. | 34.91 | 26.84 | 25.94 | 35.73 | 33.40 | 13.18 | 40.85 | 26.20 |
| significance (Dunnett) related to cells not treated with steroids (0) | | 99% | 99% | 99% | 99% | 99% | 99% | NS |
| significance (1 way analysis of variance) related to identic doses of aldosterone | | 99% | 99% | 95% | NS | NS | NS | NS |
| P | | 0.0012 | 0.0034 | 0.0144 | | | | |

*c* Effect of spironolactone + aldosterone

| Dose of steroids, g/ml | 0 | $10^{-5}$ | $10^{-6}$ | $10^{-7}$ | $10^{-8}$ |
|---|---|---|---|---|---|
| n | 7 | 7 | 4 | 7 | 4 |
| mean | 338.36 | 155.03 | 135.00 | 167.35 | 247.28 |
| ±S.D. | 34.91 | 30.25 | 26.49 | 34.30 | 19.78 |
| significance (Dunnett) related to cells not treated with steroids (0) | | 99% | 99% | 99% | 99% |
| significance (one way analysis of variance) related to identic doses of aldosterone | | 99.9% | NS | NS | 99% |
| P | | 0.0005 | | | 0.0060 |
| significance (one way analysis of variance) related to identic doses of spironolactone | | NS | NS | NS | NS |

[1] ACTH release was stimulated by 1 U/ml of Pitressin$^{R}$ in each column of the Table.
[2] Mean of 12 control incubations is subtracted from the values obtained with treated cells.

The inhibitory effect of large doses ($10^{-7}$, $10^{-6}$ and $10^{-5}$ g/ml) of aldosterone was significantly higher than that of spironolactone. Less marked was the inhibition by a combination of spironolactone and aldosterone than of aldosterone alone. The effect of the spironolactone + aldosterone combination did not differ significantly from that of spironolactone, suggesting that, when administered simultaneously with aldosterone, the effect of spironolactone prevailed.

## Discussion

Thus, a high dose of dexamethasone did not influence the basal release of ACTH in our pituitary cell-culture system, although it inhibited the stimulatory effect of α-MSH and Pitressin$^R$. Similar observations have been reported by others [2, 18, 22]: the corticotropin release stimulated by hypothalamic extracts and by potassium was suppressed by glucocorticoids, but not below the basal level. In contrast, it is known from clinical experience and animal experiments that dexamethasone decreases the plasma ACTH concentration to below its non-treated value.

The contradiction may be explained by several facts, viz. (1) the in vivo inhibitory action of the steroid may manifest itself not only directly on the pituitary but also through higher regulatory systems; (2) the value considered as the basal level in vivo is in fact a consequence of a stimulated secretion as the pituitary may continuously be influenced by various endogenous CRF-like stimuli; (3) the characteristics of the cell receptors might be altered by the enzymatic treatment of the cells during the culturing process.

ACTH stimulates the secretion of glucocorticoids, mineralocorticoids and sex hormones from the adrenal cortex. Though it is widely accepted that only the glucocorticoids play an important role in the regulation of in vivo ACTH secretion by their negative feed-back effect [1, 2, 7, 9, 18], a less pronounced inhibition has been observed in vitro with some other steroids such as aldosterone [19, 20]. Thus, our experience with aldosterone is in partial agreement with the findings of other authors, although aldosterone induced a more marked suppression of ACTH release than did dexamethasone in our cell culture system. This phenomenon may be due to certain differences in the experimental conditions.

Our finding with spironolactone deserves some attention. As a competitive antagonist of aldosterone, spironolactone is widely employed in the therapy of various diseases. Numerous effects and side-effects have been reported, for instance chronic administration of spironolactone induces secondary hyperaldosteronism; its presence inhibits the aldosterone-secretion stimulating effect of ACTH; spironolactone has progestative and antiandrogeneic actions; it disturbs the fluorimetric determination of cortisol etc. [3, 4, 5, 8, 12, 13, 17,

23, 25]. We have demonstrated a new effect of spironolactone in vitro: it inhibits the release of ACTH. However, the limited conclusiveness of the rat pituitary cell culture technique employed in our experiments has to be considered. Accordingly, the significance of these findings in clinical use still remains to be investigated.

## Acknowledgements

The authors wish to thank Dr. N. Ling, San Diego, California, for the synthetic α-MSH preparation; Parke-Davis, Montreal, Que., Canada, for the Pitressin (posterior pituitary extract) preparation; and Dr. P. Vecsei, Heidelberg, FRG, for the antiserum used in the RIA of ACTH. This work was supported by the Medical Research Council of Canada and by the National Cancer Institute of Canada.

## REFERENCES

1. Brattin, W. J., Portanova, R.: Corticosterone induction of pituitary hyporesponsiveness to CRF: divergent effects of puromycin and cyclohexamide. Mol. Cell. Endocrinol. **7,** 221—231 (1977)
2. Brattin, W. J., Portanova, R.: Effect of cordycepin on CFR stimulation and steroid inhibition of ACTH secretion by rat pituitary cells. Mol. Cell. Endocrinol. **9,** 279—289 (1978)
3. Conn, J. W., Hinerman, D. L.: Spironolactone-induced inhibition of aldosterone biosynthesis in primary aldosteronism. Morphological and functional studies. Metabolism **26,** 1293—1307 (1977)
4. Elias, A. N., Gwinup, G.: Effects of some clinically encountered drugs on steroid synthesis and degradation. Metabolism **29,** 582—595 (1980)
5. Gaillard, R. C., Riondel, A. M., Chabert, P., Vallotton, M. B.: Effect of spironolactone on aldosterone regulation in man. Clin. Sci. **58,** 227—233 (1980)
6. Gutkowska, J., Julesz, J., St.-Louis, J., Genest, J.: Radioimmunoassay of corticotropin from plasma. Clin. Chem. **28,** 2229—2234 (1982)
7. Hashimoto, K., Yunoki, S., Kageyama, J., Ohno, N., Takahara, J., Ofuji, T.: Vasopressin and CFR-ACTH in adrenalectomized and dexamethasone-treated rats. Neuroendocrinology **32,** 87—91 (1981)
8. Hertz, R., Tullner, W. W.: Progestational activity of certain steroid-17-spironolactones. Proc. Soc. Exp. Biol. Med. **99,** 451—452 (1958)
9. Jones, M. T., Hillhouse, E. W., Burden, J. L.: Structure-activity relationships of corticosteroid feedback at the hypothalamic level. J. Endocrinol. **74,** 415—424 (1977)
10. Julesz, J., Lis, M., Gutkowska, J., Genest, J.: New Pitressin preparation with diminished corticotropin releasing activity in vitro. Lancet **2,** 253 (1981)
11. Julesz, J., Lis, M., Gutkowska, J., Mizsei, I., Genest, J.: Inhibition of corticotropin release in vitro by dexamethasone, aldosterone and spironolactone. Submitted for publication J. Steroid Biochem. 1982
12. Kagawa, C. M.: Blocking the renal electrolyte effects of mineralocorticoids with orally active steroidal spironolactone. Endocrinology **67,** 125—132 (1970)
13. Kem, D. C., Weinberger, M. H., Higgins, J. R., Kramer, N. J., Gomez-Sanchez, C., Holland, O. B.: Plasma aldosterone response to ACTH in primary aldosteronism and in patients with low renin hypertension. J. Clin. Endocrinol. **46,** 552—560 (1978)
14. Korenman, S. G., Sanborn, B. A.: Hormonal assays employing tissue receptors. In: Principles of competitive protein-binding assays. pp. 89—107. eds.: Odell, W. D., Daughaday, W. H. Lippincott, Philadelphia, 1971
15. Lis, M., Julesz, J., Gutkowska, J., Genest, J.: Corticotropin-releasing activity of α-melanotropin. Science **215,** 675—677 (1982)
16. Lis, M., Larivière, N., Maurice, G., Julesz, J., Seidah, N., Chrétien, M.: Concomitant changes of ACTH, β-endorphin and N-terminal portion of proopiomelanocortin in rats. Life Sci. **30,** 1159—1164 (1982)
17. Mantero, F., Armanini, D., Urbani, S.: Antihypertensive effect of spironolactone in essential, renal and mineralocorticoid hypertension. Clin. Sci. **45,** suppl. 219S—224S (1973)
18. Portanova, R., Sayers, G.: An in vitro assay for corticotropin releasing factor(s) using suspensions of isolated pituitary cells. Neuroendocrinology **12,** 236—248 (1973)

19. Roberts, J. L., Johnson, L. K., Baxter, J. D., Budarf, M. L., Allen, R. G., Herbert, E.: Effect of glucocorticoids on the synthesis and processing of the common precursor to adrenocorticotropin and andorphin in mouse pituitary tumor cells. In: Hormones and cell culture. B. pp. 827—841, eds.: Sato, G. H., Ross, R. Cold Spring Harbor, 1979
20. Sabol, S. L., Ling, A., Daniels, M. P.: Endorphins of clonal pituitary tumor cells: synthesis, storage, secretion and regulation by glucocorticoids. In: Hormones and cell culture. B. pp. 843—853 eds.: Sato, G. H., Ross, R. Cold Spring Harbor, 1979
21. Vale, W., Grant, G., Amoss, M., Blackwell, R., Guillemin, R.: Culture of enzymatically dispersed anterior pituitary cells; functional validation of a method. Endocrinology **91,** 562—572 (1972)
22. Vale, W., Rivier, C.: Substances modulating the secretion of ACTH by cultured anterior pituitary cells. Fed. Proc. **36,** 2094—2099 (1977)
23. Vallotton, M. B., Riondel, A. M., Gaillard, R., Guelpa-Decorzant, C.: Effect of spironolactone administration on plasma and urinary aldosterone in man and the rat. Progr. Biochem. Pharmacol. **17,** 58—65 (1980)
24. Vinson, G. P., Whitehouse, B. J., Dell, A., Etienne, T., Morris, H. R.: Characterisation of an adrenal zona glomerulosa stimulating component of posterior pituitary extracts as α-MSH. Nature **284,** 464—467 (1980)
25. Wade, A.: Martindale: The extra pharmacopoeia 27th Ed, London, 1978

*Acta Medica Hungarica, 41 (1), pp. 43—54 (1984)*

# TRITIATION OF VASOPRESSIN ANALOGUES AND THEIR METABOLIC FATE AFTER INTRAVENOUS INJECTION IN THE RAT

T. JANÁKY, F. A. LÁSZLÓ and J.—L. MORGAT[1]

ENDOCRINE UNIT AND RESEARCH LABORATORY, FIRST DEPARTMENT OF MEDICINE, UNIVERSITY MEDICAL SCHOOL, H-6701 SZEGED P.O.B. 469, HUNGARY, AND [1]INSTITUTE OF BIOCHEMISTRY, NUCLEAR RESEARCH CENTRE, SACLAY, FRANCE

Received: February 28, 1983

Tritiated vasopressin analogues (LVP, AVP, dDAVP) of high specific activity were produced. The labelled peptides were purified by RP-HPLC. The biological half-life, organ distribution and in vivo metabolism of the hormones were studied in the rat. The half-life of dDAVP was longer than that of LVP or AVP. The half-life calculated from [$^3$H]AVP isolated from bloods was shorter than the value based on total radioactivity. The hormones were degraded in the kidney, the liver and small intestine, the main metabolic product being [$^3$H]Tyr. Large radioactivity accumulations were detected in the neurohypophysis and the adenohypophysis.

**Keywords:** tritiated vasopressin analogues, half-life, organ distribution, metabolism

## Introduction

Experimental endocrinology first defined the physiological significance of the neurohypophyseal hormones. Vasopressin and oxytocin are nonapeptides which play a role in the regulation of a number of vital processes. To clarify the mechanism of action of vasopressin, we felt it necessary to establish its manner of disappearance from the blood circulation; we also wished to collect data on its metabolism in vivo. It is difficult to perform such experiments because of the low hormone levels and the rapid enzymatic degradation; this necessitates the use of a considerable multiple of the physiological hormone concentration. Investigation of these processes by bioassay is complicated and inaccurate [1, 5, 9, 12, 33], while studies involving radioimmunoassay without a sufficiently specific antiserum failed to give reliable results [20, 31, 39]. Work at the molecular level was greatly aided by the availability of radiolabelled peptides of high specific radioactivity. A physiological vasopressin concentration has been attained with iodine labelled vasopressin [2, 20, 35, 36, 43], but the biological activity of the iodinated peptide was different from that of the unlabelled hormone. In experiments with tritiated vasopressins, although the employed amount of hormone was higher than the physiological level, these compounds had full biological activity [15, 22, 36, 38, 40, 43, 45].

Send offprint requests to Dr. T. Janáky, H-6701 Szeged, P.O.B. 469.

Earlier attempts to label vasopressin with tritium started with the application of the Wilzbach method [11]. A more advanced approach was the incorporation of a labelled amino acid ([$^3$H]Tyr, [$^{14}$C]Gly) into the hormone by classical techniques of peptide synthesis [37, 41]. Now, catalytic dehalogenation is the usual method of producing tritiated neurophyseal hormones of high specific activity [10, 16, 18, 29].

The present paper reports on the tritiation of vasopressin analogues via their diiodo derivatives, and on the organ distribution and disappearance from the circulation of intravenously injected [$^3$H]8-lysine-vasopressin ([$^3$H]LVP), [$^3$H]8-arginine-vasopressin ([$^3$H]AVP) and [$^3$H]1-deamino-8D-arginine-vasopressin ([$^3$H]dDAVP).

## Materials and methods

### *Radioactive materials*

The tritiated vasopressin analogues were prepared by catalytic tritiation from dihalogenated tyrosine-containing synthetic vasopressin derivatives [16]. 3,5-Dibromotyrosine$^2$-8--lysine vasopressin was synthetized by the conventional solid-phase method. After tritiation the peptide was purified in two consecutive steps by Sephadex G-15 gel-chromatography [18].

Synthesis of 3,5-diiodotyrosine$^2$-8-argini ne-vasopressin and 3,5-diiodotyrosine$^2$-1-deamino-8D-arginine-vasopressin was carried out by the method of Flouret et al. [10] with some modification [16]. The reaction products were purified by reversed-phase high-performance liquid chromatography (HPLC) [17].

1 $\mu$mol diiodo-AVP or diiodo-dDAVP was tritiated in 0.1 M phosphate buffer pH 6.0 in the presence of 15 mg $Pd/Al_2O_3$ catalyst during 20 min, in a special-purpose tritiating apparatus [27]. After purification on a Sep-pak $C_{18}$ cartridge, the tritiated peptides were separated by reversed-phase HPLC.

The specific radioactivities of the labelled hormones were determined by their UV spectra and amino acid analysis.

During the experiments, the dilute acid solutions of the tritiated peptides were stored in liquid nitrogen.

### *High-performance liquid-chromatography*

Purification of the iodinated and tritiated vasopressins was carried out on an ALTEX HPLC apparatus, consisting of two pumps (Model 110A), a System Controller (Model 420) and a Rheodyne injector (Model 7120). Column eluates were monitored for UV absorbance at 254 or 278 mm, using an ISCO Absorbance Monitor (Model 1800). The metabolic products of the labelled peptides in blood and the tissues were analysed by a Waters 6000A pump, a U6K universal liquid-chromatographic injector and an LKB Uvicord III fixed-wavelength UV monitor. Chromatography of the peptides was performed using a $\mu$Bondapack $C_{18}$ column (3.9 mm$\times$30 cm) and a Nucleosil $5C_{18}$ column (4.6 mm$\times$25 cm). All solutions were prepared with glass-distilled and deionized water, and passed through a 0.45 $\mu$m Millipore HA membrane filter. Analytical grade methanol (E. Merck, Darmstadt, FRG) was filtered on a 0.5 $\mu$m Millipore FH filter. The ammonium acetate buffer was 0.05 M in total acid and was freshly prepared by titration with ammonia solution to the desired pH. The iodinated and tritiated peptides were purified by gradient elution, while isocratic chromatography (pH 6.5 buffer — methanol = 60 — 40; v/v) was used in the metabolic experiments. Just before chromatography, the mobile phase was degassed for 5 min in an ultrasonic bath. Fractions were collected, and the radioactivity was measured in Luma-Gel liquid scintillation fluid (LUMAC B.V., The Netherlands), using a Packard liquid scintillation spectrometer (Model 3255). Quench correction was carried out with external standard ratios.

*Determination of biological half-life*

For investigation of the disappearance of the radioactivity from rat plasma, male R-Amsterdam rats weighing 220—250 g were anaesthetized with ether. A polyethylene cannula was inserted into the right carotid and diluted heparin was injected. The femoral vein was exposed and 15 $\mu$Ci (555 KBq) tritiated vasopressin analogue in a volume of 200 $\mu$l was administered. A stop-watch was started when half of the radioactive sample had been injected. Blood samples were then taken through the carotid cannula after 20 and 40 s and 1, 2, 4, 8, 16, 32 and 60 min. The plasma was obtained by microcentrifugation; 50 $\mu$l was added to 10 ml Insta-Gel liquid scintillation fluid (Packard Instrument) and the radioactivity was determined.

To establish whether the measured radioactivity had originated from the [$^3$H]AVP itself or from some metabolite, the in vivo clearance of the labelled 8-AVP was also determined in another way. After a single injection of 15 $\mu$Ci [$^3$H]AVP, blood obtained at various intervals was extracted and analysed by HPLC. It was established what percentage of the radioactivity originated from the intact peptide.

For calculation of the biological half-life, the logarithms of the radioactivities measured after different intervals were plotted versus time. A plot of the disappearance time proved to be a multiexponential curve. The curve appeared linear in the interval 8—60 min and these values were used to calculate a regression line by least-squares analysis. When the extrapolated curve was subtracted from the original curve, point by point, a second linear curve was resolved. The half-life ($t_{1/2}$) was calculated from the slope of the straight line relating the natural logarithm of the radioactivities to time ($t_{1/2} = \ln 2/\text{slope}$).

*Organ distribution*

One hour after the administration of tritiated peptide, the animals were decapitated and the organs were rapidly removed. The smaller organs in their entirety, and 100 mg tissue from the larger organs, were dissolved in Soluene-350 (Packard Instrument), and the radioactivities were measured. The results were expressed as percentages of the total radioactivity added, measured per 100 mg wet organ weight.

In those organs in which the metabolism of vasopressin occurs (kidney, liver, small intestine), a study was made of the extent to which the accumulated radioactivity originated from the undecomposed [$^3$H]AVP. The [$^3$H]AVP (15 $\mu$Ci) was administered through the femoral vein to urethan-anaesthetized rats. At 2, 5 and 60 min one of the kidneys, one lobe of the liver, and a portion of the small intestine were removed simultaneously from the animals and immediately placed in liquid air. Samples from these organs were homogenized, the proteins were precipitated and supernatants were analysed by HPLC by the method described above.

The radioactivity of urine taken from the bladder at the end of the [$^3$H]AVP experiment was determined in a similar manner.

## Results

In the iodination of AVP and dDAVP, the reaction product was 95% diiodinated peptides accompanied by a small amount of unreacted vasopressin, monoiodinated vasopressin and other, unidentified peptides. The diiodopeptides were easily separated from other products by reversed-phase HPLC. The yield of iodination and purification exceeded 80%. The purities of these diiodo-vasopressin analogues were checked by HPLC, TLC and their UV spectra. The iodination of tyrosine shifted the peptide spectra to longer wavelength:

$$\lambda_{max}^{H_2O} = 286 \text{ mm, shoulder } 294 \text{ mm, } \lambda_{max}^{OH^-} = 310.5—311 \text{ nm.}$$

Figure 1 demonstrates the separation of the reaction products of tritiation of AVP by the gradient mode of HPLC. Four peaks could be detected: A: [diAla$^{1,6}$]-vasopressin, B: vasopressin, C: monoiodovasopressin, D: diiodovaso-

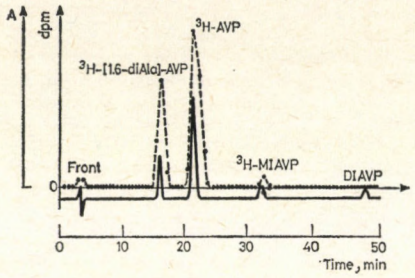



*Fig. 1.* Separation of peptides on μBondapack $C_{18}$ HPLC column after tritiation of diiodo-8--arginine-vasopressin. Elution was performed isocratically from 0 to 10 min (10% methanol in 8% acetic acid), following a linear gradient between 10% methanol in dilute acetic acid and 50% methanol in the same solution (10—50 min), at a flow rate of 1 ml/min. The eluate was monitored at 278 nm

pressin. The amino acid composition of the compound in the first peak was determined by amino acid analysis.

The specific radioactivities of the peptides used in the biological experiments were 3.5 Ci/mmol for LVP, 8.5 Ci/mmol for AVP, and 16 Ci/mmol for dDAVP (Fig. 2).

The homogeneity of each of the radioactive hormones was investigated prior to and after the experiments. Checking of the [$^3$H]LVP was performed by silica gel thin-layer chromatography. After scanning, one peak became visible, it coincided with the spot of standard LVP developed with ninhydrin (Fig. 3A). The chromatographic purity of the tritiated AVP was 97.2%, as determined by HPLC. The retention time of [$^3$H]AVP was 11.2 min, the same as for synthetic AVP (Fig. 3B). The radiochemical purity of [$^3$H]dDAVP was investigated by HPLC. The retention time of the tritiated dDAVP was 18 min, which is equal to the value for the non-labelled compound (Fig. 3C).

It may be concluded from Table I that the biological activities of the tritiated peptides are the same as those of the non-labelled hormones. After completion of the experimental series, the antidiuretic activity exhibited a moderate decrease in every case.

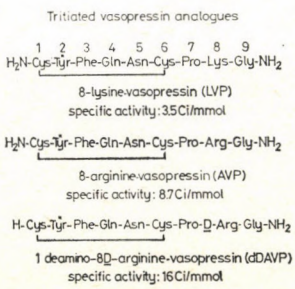



*Fig. 2.* Structures and specific radioactivities of tritiated vasopressin analogues

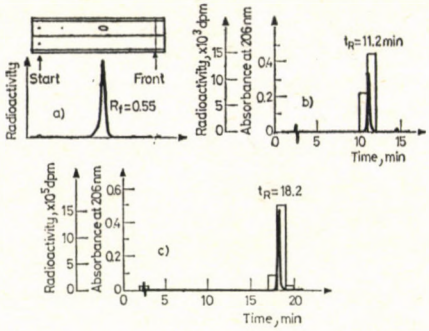



*Fig. 3.* Radiochemical purities of the labelled peptides. *A:* [$^3$H]LVP: silica gel TLC sheet; chromatographic system: n-butanol — water — acetic acid (8 : 5 : 4, v/v/v). *B:* [$^3$H]AVP: Nucleosil $5C_{18}$ HPLC column; elution system: ammonium acetate buffer (0.05 M, pH 6.5) — methanol (60 : 40, v/v); flow rate 1.2 ml/min *C:* [$^3$H]dDAVP: HPLC was performed as for [$^3$H]AVP

**Table I**

*Biological activities of tritiated vasopressin analogues*

| Compound | Biological activity (I.U./mg) | | |
|---|---|---|---|
| | Non-labelled (cold) | Tritiated peptides | |
| | | before experiments | after |
| 8-lysine-vasopressin | 270.0±17.5* | 267.5±18.5 | 248.3±21.4 |
| 8-arginine-vasopressin | 411.3±26.7 | 397.2±19.9 | 380.9±21.6 |
| 1-deamino-8*D*-arginine-vasopressin | 955.1±37.5 | 928.0±47.3 | 907.4±45.3 |

* mean ±S.E.M.

These results indicated that the compounds were biologically active and radiochemically homogeneous.

For investigation of the disappearance of radioactivity from the rat plasma the activities measured at individual points of time were plotted separately for each animal on semilogarithmic paper. The plot followed an at least double exponential function, with a rapid distribution component (fast phase) and a slower clearance component (slow phase). The typical disappearance curve of tritiated vasopressin in a rat is shown in Fig. 4.

Table II shows the mean half-life of the labelled vasopressin analogues in ten rats. The half-life of AVP and dDAVP barely differed in the fast phase, while the value for LVP slightly less. The half-life calculated for the slow phase was almost the same for LVP and AVP, but it was significantly longer for dDAVP.

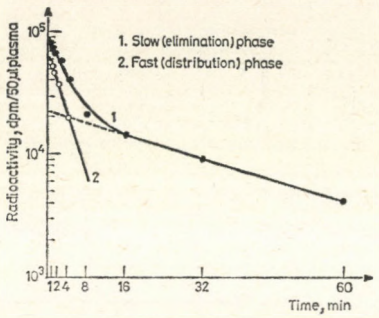



*Fig. 4.* Disappearance of radioactivity from the plasma of a rat after intravenous administration of tritiated vasopressin. Line 1 denotes the elimination (slow) phase; line 2 shows the distribution (fast) phase

**Table II**

*Biological half-lives of vasopressin analogues in R-Amsterdam rat*

| Compound | Fast phase (min) | Slow phase (min) |
|---|---|---|
| 8-lysine-vasopressin | 1.21 ± 0.07*y* | 26.55 ± 6.11 |
| 8-arginine-vasopressin | 2.49 ± 0.51 | 27.90 ± 5.08 |
| 1-deamino-8*D*-arginine-vasopressin | 2.15 ± 0.22 | 48.78 ± 8.62 |

* mean ± S.E.M.

HPLC analysis of blood after the injection of [$^3$H]AVP into the rat showed that initially vasopressin was metabolized rapidly; apart from that of AVP, two additional radioactive peaks appeared after only 30 s. One eluted with the solvent front this was proved by thin-layer chromatography to be [$^3$H]tyrosine and the size of the peak increased with time, while the other peak diminished. The quantity of [$^3$H]AVP decreased with time. From these data the biological half-life of intact vasopressin was 1.74 ± 0.22 (SD) min (n = 3) in the fast phase and 16.98 ± 1.01 min in the slow phase. These half-lives were shorter than the values calculated from the total radioactivity.

Figure 5 contains data relating to the organ distribution of the radioactivities 1 hour after the administration of tritiated vasopressin analogues to rats. In the case of [$^3$H]LVP, activity was highest in the neuro- and the adenohypophysis and in the kidney, lower in the small intestine and the liver, and very low in the muscle, the hypothalamus and the cerebral cortex. After injection of [$^3$H]AVP, most of the activity accumulated in the adenohypophysis, the small intestine, the neurohypophysis and the kidney. Activity was minimal in the cerebral cortex, the hypothalamus, and the muscle. When [$^3$H]dDAVP was given, activity was highest in the kidney, followed in turn by the small

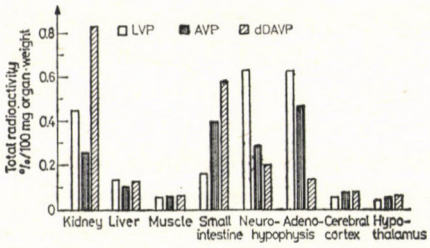



*Fig. 5.* Organ distribution of the radioactivity after [$^3$H]LVP, [$^3$H]AVP and [$^3$H]dDAVP administration to rats

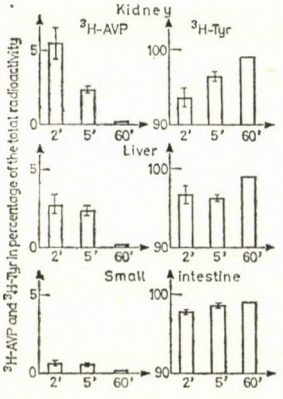



*Fig. 6.* Distribution of radioactivity in time as percentage of total radioactivity in organs (three animals)

intestine, the liver the neuro- and the adenohypophysis. Activity was very low in the muscles, the hypothalamus and the cerebral cortex.

HPLC revealed that after one hour the radioactivity in the tissues originated not from unchanged [$^3$H]AVP, but from [$^3$H]tyrosine. The data obtained after 2 and 5 min indicated that the vasopressin had undergone inactivation most rapidly in the small intestine, followed in turn by the liver and the kidney (Fig. 6). Of the radioactive substances, only [$^3$H]AVP and [$^3$H]Tyr were present.

One hour after administration of [$^3$H]dDAVP, 20—25% of the activity measured in the kidney and 0—5% of that in the liver and the small intestine originated from unchanged [$^3$H]dDAVP.

## Discussion

Investigations of the metabolic fate of circulating vasopressin have been hindered by the lack of reliable methods for hormone measurement. In contrast with the bioassay and radioimmunoassay employed in this study, experiments

involving labelled vasopressin yielded much more information. Although the labelling of vasopressin with $^{125}I$ and $^{131}I$ resulted in products of high specific activity, and the use of these tracers allowed investigations to be carried out with concentration levels approximating the physiological levels of the hormones, iodination means a loss in biological activity [2, 11]. There are differences in the clearance of unlabelled and monoiodinated vasopressin. The reduced clearance of iodinated AVP might be due to its biological inactivity [43], due to steric hindrance caused by the addition of iodine atom to a functionally important portion of the vasopressin molecule.

The incorporation of tritium atom(s) into the vasopressin abolished the difference characteristic of the labelled and non-labelled hormones. The catalytic tritiation of diiodo-Tyr-vasopressin was a convenient way to produce $^{3}H$-labelled peptides with 10—25 Ci/mmol specific activity [10, 16, 29]. Reversed-phase HPLC proved to be a method suitable for the preparation of tritiated vasopressins and allowed a rapid and complete separation of iodinated and tritiated peptides from other products.

8-Lysine-vasopressin [22, 38, 45], 8-arginine-vasopressin [15, 36, 43] and 1-deamino-8D-arginine-vasopressin [23] were tritiated by different methods and examined in biological experiments. Although a hormone concentration higher than the natural one was used in these investigations, the biological activities were complete. When considering the metabolism of the hormones, it is theoretically necessary to take into account the excretion from the circulation, the binding to plasma proteins, the outflow into the tissues, the accumulation there, and enzymatic inactivation. The vasopressin disappeared rapidly from the circulation when it had been injected intravenously. In experiments in vitro the plasma of normal rats decomposed the vasopressin slowly, and thus it is probable that the plasma does not participate directly in the metabolism of the hormone [8, 14]. There have been some earlier investigations of the biological half-life of vasopressin [1, 7, 9, 12, 13, 15, 22, 23, 25, 35, 36, 43], but species differences, the inaccuracy of the bioassays, the insufficient specificity of the radioimmunoassays, turnover differences between the analogues, the different conditions of hydration, differences in the doses administered and the different means of determination and half-life calculation make it difficult to compare the results. The disappearance of vasopressin from the plasma followed a multiexponential curve, suggesting a multicompartmental system. The rapid component is presumably saturable and receptor-mediated, while the slow component involves non-saturable, non-receptor-mediated processes. According to Weitzman and Fisher, vasopressin clearance correlated with its antidiuretic activity: the biologically inactive monoiodinated AVP disappeared more slowly than did AVP [43]. In our results the half-life in the slow phase of the biologically more active dDAVP was longer than that of LVP or AVP. The higher antidiuretic activity of dDAVP could thus be due to a higher receptor affinity, an

increased resistance to degradation of the peptide or a decreased clearance from the circulation, or their combination [7, 34].

It is obvious that estimation of the total activity measures not only the intact [$^3$H]vasopressin, but also the labelled metabolic product(s) in the blood, and thus it is not useful to assess intact hormone turnover. The undecomposed blood vasopressin level falls very rapidly after intravenous administration of the hormone. It is likely that the disappearance during the first 2 min is due to reversible migration outwards into a space approximating the extracellular fluid volume, as well as to irreversible inactivation [38]. This concept is supported by our finding that the vasopressin level extrapolated to zero time is approximately one-third of the value expected on the basis of the plasma volume, while inactivation is also fast: 2 min after injection, of [$^3$H]AVP, only 58% of the plasma activity persists in the form of undecomposed vasopressin. The discrepancy between the biological half-lives calculated from the disappearance of the total activity and the decrease in the intact AVP level may be due to the different metabolic clearances of [$^3$H]AVP and its metabolites.

One hour after the injection of tritiated vasopressins we found the following values for the non-metabolized hormones: 0—10% for LVP, 0—10% for AVP and 25—35% for dDAVP. Desamination is an important structural change, as removal from the vasopressin molecule of the amino group on the hemicysteine in position 1 decreases the rate of splitting of the Cys$^1$-Tyr$^2$ bond by aminopeptidase [32, 34, 42].The larger and more prolonged antidiuretic effect of dDAVP can be explained by the longer biological half-life.

The radioactivity in the blood not originating from the hormones must be due to the metabolism of the hormones in the tissues and the subsequent release of the metabolites into the blood. Our study of the accumulation of radioactivity in the tissues after the injection of tritiated peptides showed that activity was high in those organs (kidney and liver) in which the vasopressin was quickly metabolized. Attention must however be drawn to the considerable accumulation in the small intestine, an amount exceeding that in the liver. It was shown earlier that an extract of the small intestinal mucosa destroyed the pressor activity of vasopressin; it thus contains vasopressin-degrading enzyme(s) [21]. HPLC after 2 and 5 min indicated that the inactivation of AVP was fastest in this organ. The high accumulation of activity in the kidney suggested that metabolic activity was more marked in the kidney than in the liver and thus, in agreement with the observations of other authors, the bulk of the hormone is removed from the circulation by the kidney [26, 30]. It has oeen shown that vasopressin is secreted in the distal nephron and readsorbed br degraded in the proximal nephron [19]. The rate of inactivation is more pronounced in the liver than in the kidney [6]; this is supported by our radiochromatograms taken after 2 and 5 min: after 2 min the intact [$^3$H]AVP accounted for 5.4% of the total activity in the kidney, but only 2.7% in the

liver. The rate of degradation was the same for dDAVP, and the results confirmed the finding that this analogue was more stable than the natural AVP. The phenomenon may be explained by different enzymatic activities or by the fact that there are specific vasopressin receptors in the kidney, whereas only non-specific membrane binding has been found in the liver [30].

The neurohypophyseal hormones vasopressin and oxytocin are stored together with their respective neurophysins [4]. It is natural that a large amount of activity was observed in the neurohypophysis in the case of LVP and AVP. The binding between neurophysin and vasopressin results from the ion-pair interaction of the α-amino group of the first amino acid of the hormone and the free carboxyl group of the protein [3]. The presence of the α-amino group is therefore indispensable from the aspect of binding; the 1-deamino derivative dDAVP is unable to bind to neurophysin. [$^3$H]dDAVP uptake by the neurohypophysis was considerably less than that of [$^3$H]LVP or [$^3$H]AVP. This finding supports the inability of neurophysin to bind to dDAVP. It is noteworthy that large radioactivity accumulations were found in the adenohypophysis after administring [$^3$H]LVP or [$^3$H]AVP. The phenomenon agrees with the concept that vasopressin, as one of the corticotrophin releasing factors, may play a role in the enhancement of adrenocorticotrophin secretion [46]. This hypothesis is supported by our observation that of tritiated dDAVP, which does not exhibit a corticotrophin releasing effect [24], less is accumulated in the adenohypophysis than of LVP or AVP. The present data are in accordance with earlier studies [45] in which considerable activities were measured in the neuro- and the adenohypophysis after the intravenous administration of [$^3$H]LVP. The measured radioactivity is probably not due to intact vasopressin, as there are vasopressin-inactivating aminopeptidase and trypsin-like enzymes in the neurohypophysis [28].

## REFERENCES

1. Aziz, O.: Versuche am gekreutzen Kreislauf mit wachen Ratten. II. Endogene Sekretion und Plasmakonzentration von ADH. Eur. J. Physiol. Pflügers Arch. **311,** 355—372 (1969)
2. Baumann, G. and Dingman, J. F.: Distribution, blood transport and degradation of antidiuretic hormone in man. J. clin. Invest **57,** 1109—1116 (1976)
3. Breslow, E.: On the mechanism of binding of neurohypophyseal hormones and analogs to neurophysins. Ann. N. Y. Acad. Sci. **248,** 423—441 (1975)
4. Breslow, E., Aanning, H. L., Abrash, L. and Schmir, M.: Physical and chemical properties of the bovine neurophysins. J. biol. Chem. **246,** 5179—5188 (1971)
5. Czaczkes, J. W. and Kleeman, C. R.: The effect of various states of hydration and the plasma concentration on the turnover of antidiuretic hormone in mammals. J. clin. Invest. **43,** 1649—1658 (1964)
6. Dicker, S. E. and Greenbaum, A. L.: Inactivation of the antidiuretic activity of vasopressin by tissue homogenates. J. Physiol. (Lond.) **132,** 199—212 (1956)
7. Edwards, C. R. W., Kitau, M. J., Chard, T. and Besser, G. M.: Vasopressin-analogue DDAVP in diabetes insipidus; clinical and laboratory studies. Brit. med. J. **3,** 375—378 (1973)

8. Fabian, M., Forsling, M. L., Jones, J. J. and Lee, J.: The release, clearance and plasma protein binding of oxytocin in the anaesthetized rat. J. Endocr. **43,** 175—189 (1969)
9. Fabian, M., Forsling, M. L., Jones, J. J. and Pryor, J. S.: The clearance and antidiuretic potency of neurohypophysial hormones in man, and their plasma binding and stability. J. Physiol. (Lond.) **204,** 635—668 (1969)
10. Flouret, G., Terada, S., Yang, F., Nakagawa, S. H., Nakahara, T. and Hechter, O.: Iodinated neurohypophyseal hormones as potential ligands for receptor binding and intermediates in synthesis of tritiated hormones. Biochemistry **16,** 2119—2124 (1977)
11. Fong, C. T. O., Silver, R., Christman, D. R. and Schwartz, I. L.: On the mechanism of action of the antidiuretic hormone (vasopressin). Proc. nat. Acad. Sci. USA **46,** 1273—1277 (1960)
12. Gazis, D. and Sawyer, W. H.: Estimation of plasma half-lives of vasopressin analogs from vasopressor responses by curve-fitting. Proc. Soc. exp. Biol. Med. **157,** 584—589 (1978)
13. Ginsburg, M. and Heller, H.: The clearance of injected vasopressin from the circulation and its fate in the body. J. Endocr. **9,** 283—291 (1953)
14. Heller, M. and Zaidi, S. M. A.: The metabolism of exogenous and endogenous antidiuretic hormone in the kidney and liver in vivo. Brit. J. Pharmacol. **12,** 284—292 (1957)
15. Janáky, T., László, F. A., Sirokmán, F. and Morgat, J.-L.: Biological half-life and organ distribution of [$^3$H]8-arginine-vasopressin in the rat. J. Endocr. **93,** 295—303 (1982)
16. Janáky, T., Roy, J., Morgat, J-L. and László, F. A.: Tritiation and HPLC purification of oxytocin and vasopressin analogues. J. Label. Comp; submitted for publication.
17. Janáky, T., Tóth, G., Penke, B., Kovács, K. and László F. A.: Iodination of peptide hormones and purification of iodinated peptides by HPLC. J. Liqu. Chrom. **5,** 1499—1507 (1982)
18. Kéri, Gy. and Teplán, I.: Specific tritium labelling of lysine-vasopressin in two different aminoacid residues. J. Label. Comp. **15,** 153—161 (1978)
19. Kimura, T. and Share, L.: Characterization of the renal handling of vasopressin in the dog by stop-flow analysis. Endocrinology **109,** 2089—2094 (1981)
20. Klein, A. and Roth, J.: Antidiuretic hormone metabolism studies by radioimmunoassay. Surg. Forum **18,** 289—291 (1967)
21. Larson, E.: Tolerance and fate of the pressor principle of posterior pituitary extract in anaesthetized animals. J. Pharm. exp. Ther. **62,** 346—361 (1938)
22. László, F. A.: Biological half-lives and organ distribution of $^3$H-LVP and $^3$H-DDAVP. In: Proc. 9th Hungarian Endocrinological Congress, pp. 131—140. Ed.: F. A. László, Hungarian Academy of Sciences, Budapest, 1979
23. László, F. A., Janáky, T., Baláspiri, L. and Morgat, J-L.: Biological half-life and organ distribution of [$^3$H]1-deamino-8-D-arginine-vasopressin in the rat. J. Endocr. **88,** 181—186 (1981)
24. László, F. A., Janáky, T., Julesz, J., Kárteszi, M., Makara, G. B. and Stark, E.: Effects of 8-arginine-vasopressin and 1-deamino-8-D-arginine-vasopressin on ACTH secretion in the rat. Endocr. Exp., submitted for publication
25. Lauson, H. D.: Metabolism of the neurohypophyseal hormones. In: Handbook of Physiology. Sect. 7, vol. IV. Endocrinology Part 1, pp. 287—393. Eds. E. Knobil and W. H. Sawyer. American Physiological Society, Washington, D. C., 1974
26. Little, J. B., Klevay, L. M., Radford, E. P. Jr. and McGandy, R. B.: Antidiuretic hormone inactivation by isolated perfused rat liver. Amer. J. Physiol. **211,** 786—792 (1966)
27. Morgat, J-L., Desmares, J. and Cornu, M.: Dispositif automatique de transfer de gaz (tritium, deuterium, hydrogene). J. Label. Comp. **11,** 257—264 (1975).
28. Pliska, V., Thorn, N. A. and Vilhard, H.: In-vitro uptake and breakdown of tritiated lysine-vasopressin by bovine neurohypophyseal and cortical tissue. Acta endocr. (Kbh.) **67,** 12—22 (1971)
29. Pradelles, P., Morgat, J-L., Fromageot, P., Camier, M., Bonne, D. and Cohen, P.: Tritium labelling of 8-lysine vasopressin and its purification by affinity chromatography on Sepharose bound neurophysins. FEBS, Lett. **26,** 189—192 (1972)
30. Rabkin, R., Ghazeleh, S., Share, L., Crofton, J. and Unterhalter, S. A.: Removal of immunoreactive arginine-vasopressin by the perfused rat liver. Endocrinology **106,** 930—934 (1980)
31. Robertson, G. L., Mahr, E. A., Athar, S. and Sinha, T.: Development and clinical application of a new method for the radioimmunoassay of arginine vasopressin in human plasma. J. clin. Invest. **52,** 2340—2352 (1973)
32. Sawyer, W. H., Acosta, M., Baláspiri, L., Judd, J. and Manning, M.: Structural changes in the arginine-vasopressin molecule that enhance antidiuretic activity and specificity. Endocrinology **94,** 1106—1115 (1974)

33. Schröder, R. and Rott, D.: Über die Bestimmung und das Verhalten von ADH im menschlicher Plasma. Klin. Wschr. **37,** 1175—1181 (1959)
34. Seif, S. M., Senser, T. V., Chiarochi, F. E., Davis, B. B. and Robinson, A. G.: DDAVP (1-desamino-8D-arginine-vasopressin) treatment of central diabetes insipidus-mechanism of prolonged antidiuresis. J. clin. Endocr. **46,** 381—388 (1978)
35. Shade, R. E. and Share, L.: Metabolic clearance of immunoreactive vasopressin and immunoreactive $^{131}$I vasopressin in the hypophysectomized dog. Endocrinology **99,** 1199—1206 (1976)
36. Silver, L., Schwartz, I. L., Fong, C. T. O., Debons, A. F. and Dahl, L. K.: Disappearance of plasma radioactivity after injection of $^{3}$H-or $^{131}$I-labelled arginine-vasopressin. J. Appl. Physiol. **16,** 1097—1099 (1961)
37. Sjöholm, I. and Carlsson, L.: Synthesis of specifically tritium-labelled oxytocin and lysine-vasopressin with high specific activity. J. Label. Comp. **3,** 3—16 (1967)
38. Sjöholm, I. and Rydén, G.: Half-life of oxytocin and lysine vasopressin in blood of rat at different hormonal stages. Acta pharmacol. suec. **4,** 23—30 (1967)
39. Skowsky, W. R., Rosenbloom, A. A. and Fisher, D. A.: Radioimmunoassay measurement of arginine-vasopressin in serum: development and application. J. clin. Endocr. **38,** 278—287 (1974)
40. Towbin, E. J. and Ferrell, C. B.: Stop-flow study of renal excretion of tritiated vasopressin. J. clin. Invest. **42,** 987 (1963)
41. Walter, R. and Havran, R. T.: Arginine-vasopressin, lysine-vasopressin and oxytocin, $^{14}$C-labelled in the glycine residue. Experientia (Basel) **27,** 645—646 (1971)
42. Walter, R. and Simmons, W. H.: Metabolism of neurohypophyseal hormones: considerations from a molecular viewpoint. In: Neurohypophysis pp. 167—188. Eds.: Moses, A. M. and Share, L. Karger, Basel, 1977
43. Weitzman, R. E. and Fisher, D. A.: Arginine-vasopressin metabolism in dogs. I. Evidence for a receptor-mediated mechanism. Amer. J. Physiol. **235,** (6) E591—E597 (1978)
44. Wied de, D.: A simple automatic and sensitive method for the assay of antidiuretic hormone with notes on the antidiuretic potency of plasma under different experimental conditions. Acta physiol. pharmacol. neerland. **9,** 69—81 (1960)
45. Willumsen, N. B. S. and Bie, P.: Tissue to plasma ratios of radioactivity in the rat hypothalamo-hypophyseal system after intravenous injection of $^{3}$H-lysine$^{8}$-vasopressin and $^{3}$H-mannitol. Acta endocr. (Kbh.) **60,** 389—400 (1969)
46. Yashuda, N., Greer, M. A. and Aizawa, T.: Corticotropin-releasing factor. Endocr. Rev. **3,** 123—140 (1982)

*Acta Medica Hungarica, 41 (1), pp. 55—63 (1984)*

# THE STIMULATORY EFFECT OF A NON-OPIATE BETA-ENDORPHIN FRAGMENT ON ARGININE-VASOPRESSIN RELEASE IN RATS

F. Laczi*, J. E. C. Sykes, V. M. Wiegant and D. de Wied

RUDOLF MAGNUS INSTITUTE FOR PHARMACOLOGY, MEDICAL FACULTY, UNIVERSITY OF UTRECHT, VONDELLAAN 6, 3521 GD UTRECHT, THE NETHERLANDS

Received: February 28, 1983

The effect of the non-opiate β-endorphin (βE) fragment 2—9 and related peptides on immunoreactive (IR) arginine$^8$-vasopressin (AVP) levels was studied in the rat eye plexus plasma. Additionally, the effect of βE 2—9 on AVP release from pituitary neurointermediate lobes (NILs), in vitro was studied. IR AVP levels in the eye plexus plasma increased after subcutaneous (s.c.) injection of βE 2—9. In female rats peak values were observed 2 min after the administration of βE 2—9 (10 μg/rat). Male rats were 100 times more sensitive than female rats. A dose-response study revealed a U-shaped relationship for this effect of βE 2—9 in animals of both sexes.

From structure-activity studies it appeared that βE 2—9 was the most effective fragment. Intracerebroventricular (i.c.v.) administration of βE 2—9 did not affect the IR-AVP levels in eye plexus plasma. Moreover, AVP release from the rat NILs cells in vitro was stimulated by perfusion with βE 2—9, indicating a direct effect of the peptide on the pituitary. Therefore we suggest that βE 2—9 increases AVP release by a direct action on the posterior pituitary.

**Keywords:** vasopressin, β-endorphin 2—9, eye plexus blood, in vitro, radioimmunoassay

## Introduction

β-Endorphin (β-LPH 61—91, βE) is an endogenous peptide with opioid activities. It has been shown that circulating arginine$^8$-vasopressin (AVP) levels are altered by βE. Studies of the effect of βE on AVP release have, however, produced conflicting results: both enhancement [7, 17] and inhibition [10, 16] have been reported. The non-opiate βE fragment 2—9 (βE 2—9) exerts a long-term facilitatory effect on passive avoidance behaviour in rats similar to that found after administration of AVP. The question arose whether βE 2—9 elicited its effect by modulation of AVP release by an action on the pituitary or brain cells. In the present study the effect of βE 2—9 and related peptides on immunoreactive (IR) AVP levels in the eye plexus plasma was investigated. Additionally, we studied the effect of βE 2—9 on AVP release from the pituitary neurointermediate lobes (NIL) perfused in vitro.

* On leave of absence from the Endocrine Unit and Research Laboratory, First Department of Medicine, University Medical School, Szeged, Hungary P.O.B. 469.
Send offprint requests to F. Laczi, H-6701 Szeged, P.O.B. 469.

## Materials and methods

### *Release of AVP in vivo*

Female and male rats of a random bred Wistar strain (Cpb-WU; TNO, Zeist, The Netherlands) weighing 150 g were used. The animals had free access to water and food. The animal house was illuminated from 6 a.m. to 8 p.m. Animals were handled daily before starting the experiment. They were transported from the animal house to the experimental room 1 h before the experiment. All observations were made between 9—10 h a.m. Peptides, dissolved in saline, were administered subcutaneously (s.c.) in the neck of the animal; each animal was used only once.

For intracerebroventricular (i.c.v.) injections male Wistar rats were equipped with a permanent polyethylene cannula which had been placed under Hypnorm anaesthesia into one of the lateral ventricles of the rat brain at least 5 days before the experiment. The animals were housed singly and handled repeatedly prior to the experiment. Localization of the tip of the cannula was determined at the end of the experiment by i.c.v. injection of Evans Blue and microscopical inspection of the staining of the brain-ventricular system.

For collection of eye plexus blood, rats were anaesthetized by ether for a strictly controlled period of 45 sec. Samples of eye plexus blood were taken in cooled heparinized polyethylene tubes (3). These were centrifuged at 4°C to obtain plasma. To standardize the experiments, plasma samples were always assayed by RIA on the day of blood collection.

AVP determination was carried out in unextracted eye plexus plasma using a specific radioimmunoassay (RIA) system [11]. The antiserum used was highly specific for AVP, the crossreactivity on a molar basis with oxytocin being 0.0019% and with vasotocin 0.27%. Synthetic AVP (Organon, Oss, pressor activity: 509 U/mg was used as a standard and for preparation of the tracer [6]. Plasma of homozygous diabetes insipidus rats (TNO-code: Brat(Cpb-DI) was used for the control of possible aspecific effects.

RIA data were calculated by a Hewlett Packard 104 calculator equipped with a logit curve fitting program. IR AVP levels were expressed in fmol/ml plasma.

The following peptides were used: $\beta$-endorphin 2—9 ($\beta$E 2—9, $\beta$-LPH 62—69), $\beta$-endorphin 2—5 ($\beta$E 2—5, $\beta$-LPH 62—65), $\beta$-endorphin 5—9 ($\beta$E 5—9, $\beta$-LPH 65—69), $\beta$-endorphin 2—6 ($\beta$E 2—6, $\beta$-LPH 62—66), $\beta$-endorphin 2—7 ($\beta$E 2—7, $\beta$-LPH 62—67), $\beta$-endorphin 2—8 ($\beta$E 2—8, $\beta$-LPH 62—68), $\beta$-endorphin ($\beta$E, $\beta$-LPH 61—91), des-tyrosine-$\gamma$-endorphin (DT$\gamma$E, $\beta$-LPH 62—77), des-tyrosine-$\alpha$-endorphin (DT$\alpha$E, $\beta$-LPH 62—76) and $\beta$-LPH 78—91. The peptides were obtained from Organon International b. v., Oss, The Netherlands.

### *Release of AVP in vitro*

The method used for the perfusion of isolated rat NILs cells was a variation of that described by Gillies and Lowry [8].

The NILs cells taken from male Wistar rats were stimulated with test material dissolved in perfusion medium for 3 min every 20 min. Four min fractions (2 ml) were collected into small plastic tubes containing a mixture (100 $\mu$l) of 2 M-HCl and aprotinin (Boehringer Mannheim GMBH, West Germany) to give 100 KIU/ml. Fractions were neutralized with 1.1 M-$NaHCO_3$ before RIA.

The AVP content of the incubation medium was determined by RIA. The RIA procedure used was a variation of that of Dogterom et al. [6]. The assay had a sensitivity limit of 1.09 fmol/assay tube. None of the test substances used had any effect on the RIA. Hormone release per 20 min was expressed as a percentage of the control (unstimulated) value.

### *Statistical analysis*

Nonparametric tests of significance were used, because the conditions in data under analysis met the requirements of these tests. (The data populations were not equal in variance and the level of measurement achieved a partially ordered scale.) Statistical analysis was done by Kruskal—Wallis one-way analysis of variance followed by Mann-Whitney U-test. A probability level of less than 0.05 was accepted as a significant difference.

## Results

### *Release of AVP in vivo*

In the first series of experiments the time course of the effect of βE 2—9 (10 μg s.c. per animal) on eye plexus plasma IR AVP levels was investigated. Female rats (n = 5—8) were treated with placebo (0.2 ml saline s.c.) or βE 2—9, and 2, 5, 10, 30 or 60 min later eye plexus blood samples were collected.

As shown in Figure 1 plasma IR AVP levels were significantly increased over control values at 2, 5 and 10 min, reaching a peak at 2 min after the injection of βE 2—9. The levels returned to the control values 30 min after treatment.

In the second set of experiments the dose-response relationships were determined. Female animals (n = 8—12) were injected with placebo (0.2 ml saline s.c.) or graded doses of βE 2—9 (0.0001—10.0 μg/rat s.c.). Two min later eye plexus blood samples were withdrawn. Likewise, male rats (n = 8) were treated with placebo (0.2 ml saline s.c.) or graded doses of βE 2–9 (0.01—10.000 ng/rat s.c.). Two min later eye plexus blood samples were taken.

Graded doses of βE 2—9 induced a U-shaped dose-response curve in animals of both sexes (Figs 2, 3). In female rats the maximum effect of βE 2—9 on the eye plexus plasma IR AVP levels was observed at a dose of 100 ng/per animal. Ten and 100 times higher doses of βE 2—9 also significantly increased the IR AVP levels in eye plexus plasma over the control value, however, the

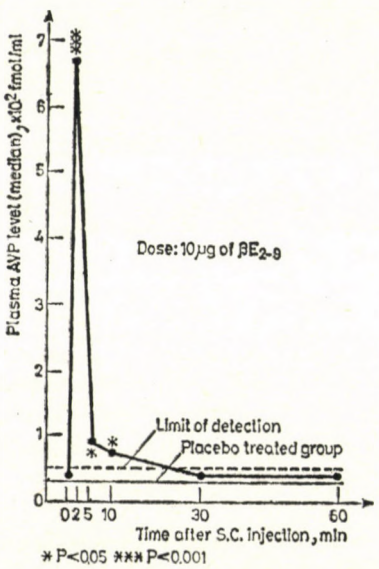



*Fig. 1.* IR AVP levels in eye plexus plasma at 2—60 min after s.c. injection of 10 μg βE 2—9. Each group contained 5—8 female Wistar rats. Asterisks indicate significant differences between βE 2—9-treated and placebo-treated groups

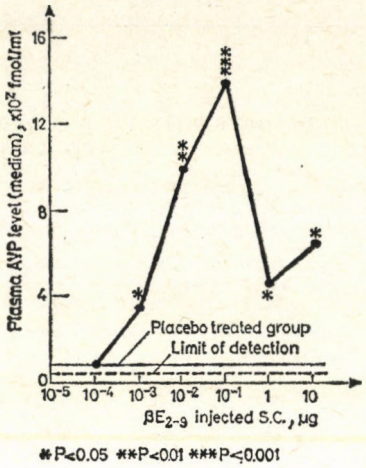



*Fig. 2.* IR AVP level in eye plexus plasma 2 min after s.c. injection of graded doses of βE 2—9 (0.0001—10.0 µg per animal). Each group contained 8—12 female Wistar rats. Asterisks indicate significant differences from placebo-treated groups

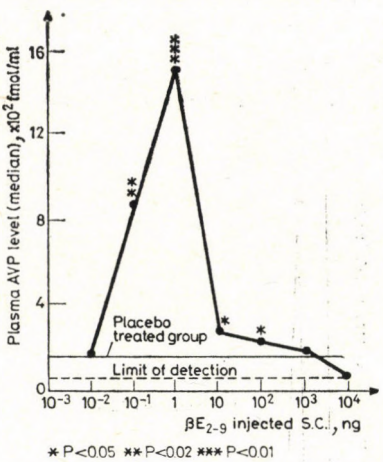



*Fig. 3.* IR AVP level in eye plexus plasma 2 min after s.c. injection of graded dose of βE 2—9 (0.01—10.000 ng per animal). Each group contained 8 male Wistar rats. Asterisks indicate significant differences from placebo-treated group

increase was smaller in extent. In male animals a maximum effect of βE 2—9 was reached at a dose of 1.0 ng per animal. At thousand and 10.000 fold higher doses of βE 2—9 the IR AVP levels in eye plexus plasma were not different from the control values.

To investigate structure-activity relationships, female rats (n = 8—22) were injected with placebo (0.2 ml saline s.c.) or 100 ng βE 2—5, βE 5—9,

βE 2—6, βE 2—7, βE 2—8, βE 2—9, DTαE, DTγE, β-LPH 78—91 or βE and 2 min later eye plexus blood samples were collected. Plasma IR AVP levels were significantly increased after administration of βE 2—9, DTαE or DTγE. From these peptides βE 2—9 was the most potent. The other βE fragments did not significantly change the IR AVP levels in the eye plexus plasma (Table I).

**Table I**

*IR AVP levels in eye plexus plasma 2 min after s.c. injection of β-endorphin (0.1 μg/rat) or its fragments (0.1 μg/rat) in female Wistar rats*

| Treatment | IR AVP (fmol/ml) | n[1] |
|---|---|---|
| Placebo | 60[2] | 22 |
| $\beta E_{2-5}$ | 270 | 8 |
| $\beta E_{5-9}$ | 104 | 8 |
| $\beta E_{2-6}$ | 72 | 8 |
| $\beta E_{2-7}$ | 67 | 8 |
| $\beta E_{2-8}$ | 174 | 8 |
| $\beta E_{2-9}$ | 1462*** | 12 |
| DTαE | 331* | 15 |
| DTγE | 470* | 13 |
| $\beta LPH_{78-91}$ | 183 | 10 |
| βE | 91 | 10 |

[1] number of animals
[2] median
* $P < 0.05$ (treatment vs placebo)
*** $P < 0.001$ (treatment vs placebo)

In a further series of experiments the effect of βE 2—9 was investigated after i.c.v. administration. Male rats (n = 5—10) bearing a permanent polyethylene cannula in one of the lateral ventricles of the brain were injected i.c.v. with placebo (1 μl artificial cerebrospinal fluid (CSF)) or βE 2—9 (0.01 or 0.1 ng/rat dissolved in 1 μl artificial CSF) and, 2, 10 or 30 min later eye plexus blood samples were obtained.

βE 2—9 did not affect the IR AVP level in eye plexus plasma in any of the experimental groups (Fig. 4).

*Release of AVP in vitro*

NIL-cells obtained from male rats were perfused with βE 2—9 at different concentrations. The peptide induced an increase in IR AVP concentration in the perfusate and βE 2—9 increased basal IR AVP release (Fig. 5). The differ-

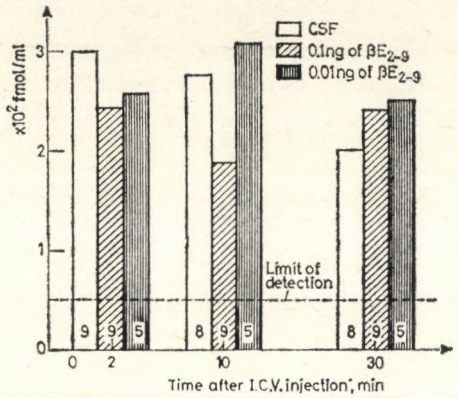



*Fig. 4.* **IR AVP level in eye plexus plasma 2, 10, 30 min after i.c.v. administration of various doses of $\beta$E 2—9 (0.01 or 0.1 ng/rat dissolved in 1 $\mu$l CSF). Number in bars represents number of male Wistar rats used**

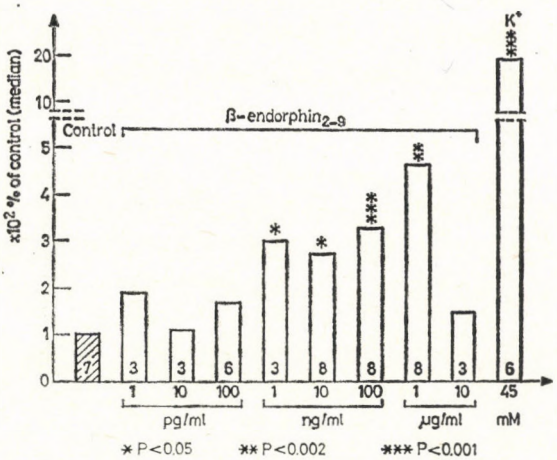



*Fig. 5.* **Effect of $\beta$E 2—9 on AVP release from neuro-intermediate lobes *in vitro*. Columns represent median value of the data expressed in per cents of the control. Number in bars shows number of stimulations with each investigated dose of $\beta$E 2—9. Asterisks indicate significant differences from control**

ences from control values were significant at doses of 1 ($P < 0.05$), 10 ($P < 0.05$), 100 ($P < 0.001$) ng/ml and 1 ($P < 0.02$) $\mu$g/ml of $\beta$E 2—9. The maximum effect of $\beta$E 2—9 (a more than 4 fold increase over the control) was observed at the dose of 1 $\mu$g/ml. A ten times higher dose (10 $\mu$g/ml) failed to increase significantly the basal AVP release. Perfusion with 45 mM KCL markedly elevated the AVP output.

## Discussion

The present study clearly indicated a powerful stimulatory effect of βE 2—9 on IR AVP secretion into eye plexus blood. The peptide had a direct effect on the posterior lobe of the pituitary gland.

The time-course of plasma IR AVP levels after a single s.c. injection of βE 2—9 (10 μg/rat) revealed a marked increase in plasma IR AVP content with a peak value at 2 min after administration of the peptide. The IR AVP levels in eye plexus plasma had returned to control values 60 min after the injection of βE 2—9, indicating a short-lasting effect of the peptide. Graded doses of βE 2—9 resulted in a U-shaped dose-response curve in animals of both sexes. De Rotte [5] investigated the effect of i.c.v. administration of des--enkephalin-γ-endorphin (DEγE) on the plasma α-melanocyte stimulating hormone (α-MSH) level and he too observed a U-shaped dose-response relationship. The reason for such a dose-response curve is not entirely clear. It is possible that high doses of βE 2—9 might inhibit the release of AVP. The peak effect of βE 2—9 on the IR AVP level in eye plexus plasma was reached at a dose of 100 ng/rat in the female animals while male rats were 100 times more sensitive in this respect. The explanation of these observations may be that sex steroids might modulate the AVP release induce by βE 2—9 from the neurohypophysis. This assumption is supported by several reports underlining the importance of sex steroids in the modulation of AVP release [1, 2, 13, 15]. Moreover, sex differences in AVP secretion have been observed during passive avoidance behaviour in rats [11]. From structure-activity studies concerning the effect of βE fragments on AVP release, it appeared that βE 2—9, DTαE and DTγE were able to augment the plasma AVP level. The most active fragment in this respect was βE 2—9. Shorter fragments of βE, i.e. βE 2—5, βE 5—9, βE 2—6, βE 2—7 and βE 2—8, appeared to be inactive. βE itself was also ineffective in increasing AVP levels in the eye plexus plasma, which is in disagreement with other observations. *In vivo* studies too have produced conflicting data on the role of βE in the control of neurohypophyseal function. Both stimulatory [7, 17] and inhibitory effects [10, 16] of βE on AVP secretion have been reported. Unlike βE, DTαE and DTγE when administered i.c.v. had no effect on the basal AVP level in peripheral plasma [16]. These findings may not necessarily be in contradiction with the present observations concerning βE, DTαE and DTγE, taking into account the differences between species, doses, the sites of administration and the time of the observation.

The N-terminal amino acid tyrosine (position 61) of βE is essential for the opiate-like activity of βE [4, 9, 14], as the removal of tyrosine caused a loss of the opiate-like activity. It is therefore assumed that the stimulatory effect of βE 2—9 on AVP secretion is not mediated by opiate receptors. I.c.v. administered doses of βE 2—9 (0.01—0.1 ng/rat) had no effect on the plasma AVP level

after 2, 10 and 30 min suggesting that the effect of βE 2—9 s.c. is mediated by a direct action on the pituitary rather than at the level of the brain. Indeed, AVP release by rat NIL cells in vitro could be stimulated by adding βE 2—9 to the perfusion medium. This indicates a direct effect of βE 2—9 on the pituitary in the regulation of AVP release.

The observation that s.c. administration of βE 2—9 did not affect blood pressure and heart rate in rats [12] excludes a reflectory AVP release induced by hypotension.

It is suggested that βE 2—9 has a powerful stimulatory effect on the plasma level of IR AVP. βE 2—9 did not seem to exert its effect by an action on the brain neither through a mechanism involving the cardiovascular system. It affects AVP release by a direct action on the pituitary. Whether or not βE 2—9 plays a physiological role in controlling the release of AVP from the neurohypophysis, has to be elucidated.

## Acknowledgements

The authors wish to thank Mr. Henk de Lang for technical assistance. This study was supported by the Stichting Pharmakologisch Studiefonds, Utrecht, the Netherlands

## REFERENCES

1. Barnafi, L., Croxatto, H.: Vasopressin and oxytocin activities in the posterior lobe of the pituitary gland of male and female rats following adrenalectomy and gonadectomy. Acta Endocrinol (Copenh) **52,** 3—6 (1966)
2. Crowley, W. R., O'Donohue, T. L., George, J. M., Jacobowitzan, D. M.: Changes in pituitary oxytocin and vasopressin during the oestrous cycle and after ovarian hormones: evidence for mediation by norepinephrine. Life Sci **23,** 2579—2586 (1978)
3. De Wied, D.: A simple automatic and sensitive method for the assay of antidiuretic hormone with notes on the antidiuretic potency of plasma under different experimental conditions. Acta Physiol Pharmacol Neerl **9,** 69—81 (1960)
4. De Wied, D., Kovács, G. L., Bohus, B., Van Ree, J. M., Greven, H. M.: Neuroleptic activity of the neuropeptide β-LPH 62—77 ([Des-Tyr$^1$]-γ-endorphin; DTγE). Eur J Pharmacol **49,** 427—436 (1978)
5. De Rotte, A. A.: Alpha-melanocyte stimulating hormone in blood and cerebrospinal fluid of the rat. pp. 133—152. Ph. D. Thesis, Utrecht, 1982
6. Dogterom, J., Van Wimersma Greidanus, Tj. B., De Wied, D.: Vasopressin in cerebrospinal fluid and plasma of man, dog and rat. Amer J Physiol **234,** E463—E467 (1978)
7. Firemark, H. M., Weitzman, R. E.: Effects of β-endorphin, morphine and naloxone on arginine vasopressin secretion and the electroencephalogram. Neuroscience **4,** 1895—1902 (1979)
8. Gillies, G. E., Lowry, P. T. J.: Perfused isolated rat anterior pituitary cell column as bioassay for factor(s) controlling the release of corticotropin: validation of a technique. Endocrinology **103,** 521—527 (1978)
9. Hambrook, J. M., Morgan, B. A., Rance, M. J., Smith, C. F. C.: Mode of deactivation of the encephalins by rat and human plasma and rat brain homogenates. Nature **262,** 782—783 (1976)
10. Knepel, W., Nutto, D., Hertting, G.: Evidence for inhibition by β-endorphin of vasopressin release during foot shock-induced stress in the rat. Neuroendocrinology **34,** 353—356 (1982)
11. Laczi, F., Fekete, M., De Wied, D.: Antidiuretic activity and immunoreactive arginine vasopressin levels in eye plexus blood during passive avoidance behavior in rats. Life Sci **32,** 577—589 (1983)

12. Laczi, F., Sykes, J. E. C., Wiegant, V. M., Sitsen, A., De Wied, D.: $\beta$-Endorphin$_{2-9}$ enhances arginine-vasopressin release by a direct action upon the neural lobe (submitted for publ. to J. Clin Endocrinol.)
13. Scowsky, W. R., Swan, L., Smith, P.: Effects of sex steroid hormones on arginine vasopressin in intact and castrated male and female rats. Endocrinology **104,** 105—108 (1979)
14. Terenius, L., Wahlström, A., Lindberg, G., Karlsson, S., Ragnarsson, U.: Opiate receptor affinity of peptides related to Leu-enkephalin. Biochem Biophys Res Commun **71,** 175—179 (1976)
15. Valiquette, G., Martini, L.: Effects of orchidectomy and sex steroids on vasopressin response to dehydration. Acta Endocrinol (Copenh) **99,** 493—499 (1982)
16. Van Wimersma Greidanus, Tj. B., Van Ree, J. M. Goedemans, H. J. H., Van Dam, A. F., Andringa-Bakker, E. A. D., De Wied, D.: Effects of $\beta$-endorphin fragments on plasma levels of vasopressin. Life Sci **29,** 783—788 (1981)
17. Weitzman, R. E., Fisher, D. A., Minick, S., Ling, N., Guillemin, R.: $\beta$-Endorphin stimulates secretion of arginine-vasopressin in vivo. Endocrinology **101,** 1643—1646 (1977)

*Acta Medica Hungarica, 41 (1), pp. 65—73 (1984)*

# EFFECT OF THE VASOPRESSIN ANTAGONIST d($CH_2$)$_5$ TYR(Et)VAVP ON DIURESIS IN RAT

F. A. László, S. Csáti, L. Balázspiri and M. Manning[1]

ENDOCRINE UNIT, FIRST DEPARTMENT OF MEDICINE, AND DEPARTMENT OF MEDICAL CHEMISTRY UNIVERSITY MEDICAL SCHOOL, SZEGED, HUNGARY; [1] DEPARTMENT OF BIOCHEMISTRY, MEDICAL COLLEGE OF OHIO, TOLEDO, OHIO, U.S.A.

Received: February 28, 1983

The effect of [1-(β-mercapto-β,β-cyclopentamethylene-propionic acid)2-0-ethyl-tyrosine,4-valine] arginine vasopressin on the water metabolism was studied in rat. The compound was found to be able to block the antidiuretic action of both exogenous and endogenous vasopressin.

A rat model of the Schwartz—Bartter syndrome was created by the administration of a high dose of a posterior pituitary preparation (Pitressin tannate) together with a forced water intake. The antagonist prevented water retention and averted the enhanced natriuresis and hyponatraemia, and cerebral oedema did not develop. The observations suggest that this vasopressin antagonist might be of use in the future as an effective drug against the Schwartz—Bartter syndrome.

**Keywords:** vasopressin antagonist, antidiuretic action, Pitressin tannate, water retention, hyponatraemia, cerebral oedema

## Introduction

Hyperadiuretism was first described by Schwartz et al. in 1957 in connection with a case of bronchial carcinoma [12]. Increasingly great importance has recently been attributed to the Schwartz-Bartter syndrome, involving hypernatriuria, hyponatraemia and cerebral oedema.

Table I, prepared on the basis of the survey by Edwards [3], presents the states, diseases and drugs that can induce the syndrome. The first group relates to ectopic vasopressin formation, i.e. the paraneoplastic endocrine syndrome. The vasopressin is produced by various tumour tissues (carcinoma of the bronchus, pancreas, duodenum and ureter). Bronchial carcinoid and pulmonary tuberculosis too are included here. An enhanced vasopressin efflux may also be caused by a physiological or pathological volume stimulation. Mention may be made of ventilation at positive pressure and benign pulmonary diseases. The pressure fall in the left atrium is presumably the causal factor in postcommissurotomy; the reduction in the circulating blood volume may play a role in haemorrhage and in endocrine and congestive heart diseases. The third group contains those diseases in which the excessive vasopressin production probably

Send offprint requests to F. A. László, H-6701 Szeged P.O.B. 469

**Table I**

*(Edwards, 1971)*

Causes of Schwartz—Bartter syndrome

1. *Ectopic vasopressin formation (paraneopl. end. s.)*
   - carcinoma of bronchus, pancreas, duodenum, ureter
   - bronchial carcinoid
   - pulmonary tuberculosis
2. *Abnormal vasopressin volume stim. (phys.-path.)*
   - ventilation at pos. pressure
   - benign pulmonary disease (pneumonia, aspergillosis)
   - postcommissurotomy
   - haemorrhage
   - endocrine diseases (hypopit., hypothyr., hypadr.)
   - congestive cardiac diseases
3. *Nervous system stim.* → *vasopressin release*
   - cerebral (trauma, neopl. inflammation, bleeding, surgery)
   - Guillain—Barré S.
   - acute intermittent porphyria
   - drugs: clofibrate, vincristin
4. *Drugs* → *renal sens. increases to vasopressin*
   - chlorpropamide, carbamazepine
5. *Vasopressin overdose*
   - VP, dDAVP

arises as a consequence of stimulation of the nervous system. Causes other than cerebral lesions include the Guillain-Barré syndrome, acute intermittent porphyria and the drugs clofibrate and vincristin. Drugs may enhance the sensitivity of the renal tubules to vasopressin (chlorpropamide, carbamazepine). Finally, mention should be made of the possibility of vasopressin overadministration.

This listing itself demonstrates that the Schwartz-Bartter syndrome is not rare. Its adequate treatment poses an appreciable problem; water withdrawal and sodium-retaining hormone administration may be tried. Apart from the theoretical interest, therefore, it is of very great importance to prepare compounds with effects opposite to that of vasopressin as regards the influence on the water metabolism. Of the several hundred neurohypophyseal peptide analogues synthetized in the Department of Biochemistry at the Medical College of Ohio, several exhibited an antagonist effect as concerns the antidiuretic action. One of the most important of these compounds is a vasopressin analogue: [1-($\beta$-mercapto-$\beta$,$\beta$-cyclopentamethylene-propionic acid),2-O-ethyl

tyrosine,4-valine] arginine vasopressin, abbreviated as d$(CH_2)_5$Tyr(Et)VAVP. We have made a study of the influence of this compound on the water metabolism in rat, with two main aims: to establish how the susbtance acts on the antidiuretic effects of exogenous and endogenous vasopressin, and to learn whether the changes induced by the administration of a high dose of a vasopressin preparation (water retention, hypernatriuria, hyponatraemia, cerebral oedema) can be prevented with the vasopressin antagonist.

## Materials and methods

Details of the preparation of the compound have been reported by Manning et al. [6, 8]. The chemical composition is presented in Fig. 1. Compared with the structure of the vasopressin molecule, differences may be observed at three sites. The hemicysteine at position 1 is

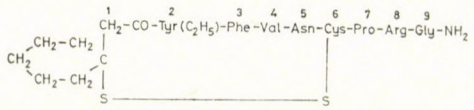



*Fig. 1.* Chemical structure of d$(CH_2)_5$Tyr(Et)VAVP

replaced by cyclopentamethylene-propionic acid; there is an ortho-ethyl group on the tyrosine in position 2; the glutamine at position 4 is replaced by valine. Preliminary experiments demonstrated that the analogue causes a considerable decrease in the antidiuretic action of vasopressin [6, 7, 11]. This property (ability) of the antagonist is characterized by the "effective dose"; this is defined as the quantity of the substance which halves the antidiuretic effect observed 20 minutes after the intravenous administration of 2 I.U. vasopressin. In the present case this proved to be 1.9 nmol/kg (1 nmol = 1.14 $\mu$g). The antagonism can not be regarded as selective. The compound also displays a marked antipressor action; Manning et al. [8] found the "effective antivasopressor dose" to be 0.49 nmol/kg.

Our first experimental series dealt with the question of how this antagonist influences the antidiuretic action of an exogenously administered vasopressin preparation. Female homozygous rats of the Brattleboro strain, weighing 160—180 g, were used; these animals do not possess an endogenous ADH reserve. Doses of 10 $\mu$U or 30 $\mu$U arginine vasopressin (Organon, Oss) were injected intravenously into animals prehydrated by the method of de Wied [1] and anaesthetized with ethanol. The antidiuretic effect was expressed as a percentage diuresis change; this was taken as the quantity of urine collected with a bladder catheter in a 10-minute period before injection of the hormone, as a percentage of the corresponding quantity after the injection. The antagonist was administered intravenously in the "effective dose" (1.9 nmol/kg = 2.16 $\mu$g/kg) 20 minutes before the injection of vasopressin.

We subsequently studied whether d$(CH_2)_5$Tyr(Et)-VAVP is able to suspend the antidiuretic effect of endogenous vasopressin, and whether it can induce a state corresponding to diabetes insipidus. Male rats of the R-Amsterdam strain, weighing 180—200 g, were used in these experiments. The animals were placed individually in cages suitable for urine collection; the amount of urine was measured in 1-hour periods for 4 hours after administration of the antagonist, and the osmolality of the urine samples was determined with and Advance osmometer. Both before and during the study the rats received water ad libitum. The antagonist was injected intravenously in a dose of 10 or 30 $\mu$g/kg, dissolved in 0.2 ml physiological NaCl. Besides the rats of the R-Amsterdam strain, homozygous Brattleboro rats too were used as controls. Instead of the antagonist, the control groups received 0.2 ml physiological saline solution intravenously.

In the following experiments, physiological NaCl solution in a dose of 5% of the body weight was administered via a stomach tube to rats of the R-Amsterdam strain, and the urinary output was measured in 1-hour periods for 4 hours. In the meantime the animals did not con-

sume liquid. This method is suitable for inducing water retention by enhancing endogenous vasopressin release [4, 5]. Instead of physiological NaCl solution, tap-water was administered to the control group. The vasopressin antagonist, in a dose of 30 μg/kg, was injected intravenously immediately before the loading with physiological NaCl or tap-water.

With a view to deciding how suitable the antagonist might be as a drug for the treatment of the Schwartz-Bartter syndrome, an attempt was made to induce a situation in rat corresponding to the human syndrome. The rats were treated daily with a large subcutaneous dose (1 I.U.) of long-acting posterior pituitary extract (Pitressin tannate; Park Davis, Munich); to attain the necessary degree of hydration, the rats also received tap-water via a stomach tube in a dose of 5% of the body weight, three times daily. Besides the Pitressin, the vasopressin antagonist too was administered subcutaneously to some of the animals, in a dose of 3×60 μg/kg b.w. During these experiments, the daily urinary output and the urinary osmolality were measured as before, and the urine and serum sodium levels were determined with a flame photometer. Following the examinations, on the 6th day of treatment the change in the water content of the brain was established by dehydration to weight constancy; the water content was calculated per 100 mg wet brain weight.

## Results

The data relating to diuresis inhibition, expressed as a percentage of the urinary output, are to be seen in Fig. 2. Arginine vasopressin decreases the urinary output in a dose-dependent manner. Administration of the antagonist before the arginine vasopressin injection moderated the diuresis inhibition considerably.

The results of the experiments aimed at suppressing the antidiuretic effect of endogenous vasopressin are given in Figs 3—5. It may be observed that a 10 μg/kg body weight dose of the antagonist caused hardly any change in the urinary output (Fig. 3), whereas the dose of 30 μg/kg body weight enhanced the diuresis appreciably. The maximum in the polyuria is to be seen in the fraction from the 2nd hour. In this period the quantity of urine attained the level for the homozygous Brattleboro rats with a total ADH deficiency. The diuretic action of the antagonist is a temporary one; after 4 h the urinary output has returned to the normal level. The urinary osmolality varies accordingly (Fig. 4). It is decreased by the vasopressin antagonist in a dose-dependent way. With this method a considerable difference can be demonstrated between

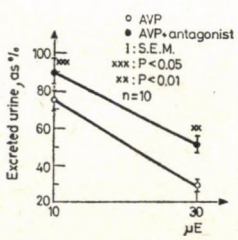



*Fig. 2.* Percentage change in urine excretion in response to vasopressin antagonist (n = 10)

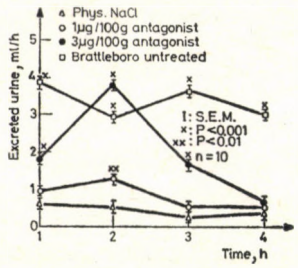



*Fig. 3.* Effect of vasopressin antagonist on urine excretion (n = 10)

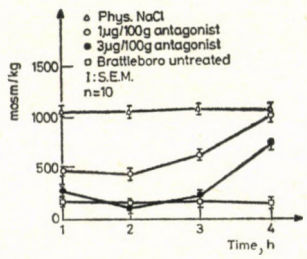



*Fig. 4.* Effect of vasopressin antagonist on urinary osmolality (n = 10)

the osmolality values for the controls and even that following administration of the low dose of the antagonist. After water loading, the antagonist enhances the diuretic reaction only moderately (Fig. 5). The administration of physiological NaCl in place of tap-water led to a marked water retention in the control group. The antagonist averted this effect completely; the extent of urine excreted in the fraction from the 2nd hour far exceeded the mean measured after tap-water loading.

A considerable water retention can be observed following administration of a large dose of the posterior pituitary extract (Fig. 6). The rats excreted

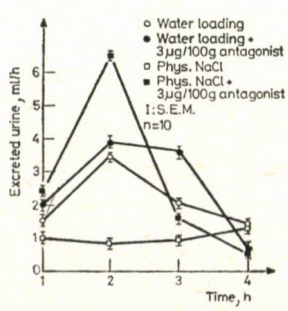



*Fig. 5.* Effect of vasopressin antagonist on diuretic reaction following water and phys. NaC loading (n = 10)

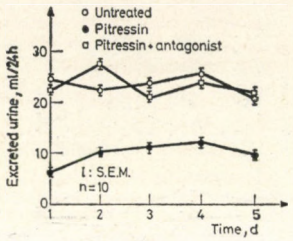



*Fig. 6.* Effect of vasopressin antagonist on urine excretion of rats treated with Pitressin (n = 10)

about one-third of the artificially administered water. With simultaneous application of the vasopressin antagonist, the water retention could be prevented. The osmolality of the urine of Pitressin-treated animals is increased to a great extent on the first day after administration of the drug (Fig. 7). The osmolality later decreases, and on the 4th—5th day of the therapy approaches the mean level for the controls. The vasopressin antagonist abolished the change in urinary osmolality; the values measured in this group did not differ from those for the control rats. Sodium excretion in the urine was expressed in mmol/24 h (Fig. 8). In response to Pitressin administration, the sodium output rose considerably; it was the highest on the day after treatment, and subsequently fell progressively, reaching the control level on the 5th day. The vaso-

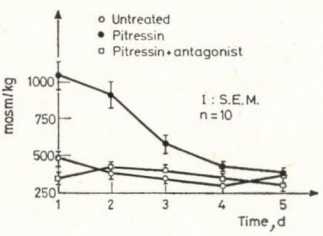



*Fig. 7.* Effect of vasopressin antagonist on urinary osmolality of rats treated with Pitressin (n = 10)

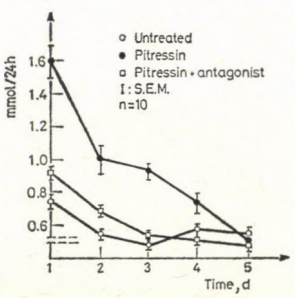



*Fig. 8.* Effect of vasopressin antagonist on sodium excretion of rats treated with Pitressin (n = 10)

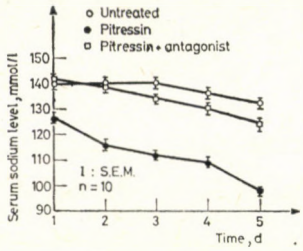



*Fig. 9.* Effect of vasopressin antagonist on serum sodium level of rats treated with Pitressin (n = 10)

pressin antagonist substantially moderated the elevated natriuresis; the sodium content of the urine did not exceed (or only slightly) the value for the untreated rats. Corresponding changes were observed in the serum sodium level (Fig. 9). On the action of the posterior pituitary extract (with forced hydration), the serum sodium level decreased on the first day of treatment. The hyponatraemia later became more marked, falling below 100 mmol/l by the end of the experimental period. The animals tolerated the hyponatraemia badly, one-third of them dying within 5 days. These data were not taken into consideration in the evaluation. In animals which received the vasopressin antagonist together with Pitressin, only a moderate serum sodium level decrease was found on the 3rd—5th days of treatment.

The serum osmolality and the cerebral water content were determined at the end of the experimental period. The results are given in Table II. In the Pitressin-treated animals the serum osmolality was decreased significantly, while the weight of the brain was higher, due to its increased water content.

**Table II**

*Effect of vasopressin antagonist on serum osmolality and water content of brain in rats treated with Pitressin*

| Groups | Number of animals | Body weight (g) | Se osmolality (mosm/kg) | Weight of brain (g) | Water content of brain (mg/100 mg wet weight) |
|---|---|---|---|---|---|
| 1 Untreated | 10 | 180.7 ± 7.1* | 285.0 ± 20.7 | 1.56 ± 0.11 | 75.0 ± 1.1 |
| 2 Pitressin | 10 | 198.2 ± 12.3 | 236.2 ± 11.3+ | 1.87 ± 0.13+ | 78.1 ± 0.6+ |
| 3 Pitressin + antagonist | 10 | 187.0 ± 10.6 | 275.4 ± 16.9 | 1.57 ± 0.10 | 74.5 ± 1.3 |

* mean ± S.E.M.
\+ $P < 0.05$ compared with untreated rats (Student's t-test)

Although the difference was not too high numerically, this latter result is indicative of the existence of a marked cerebral oedema. By means of administration of the vasopressin antagonist, these changes could be prevented completely.

## Discussion

Our results demonstrate that the vasopressin antagonist examined, $d(CH_2)_5$Tyr(Et)VAVP, is able to block the antidiuretic effect of both exogenous and endogenous vasopressin.

One of the main conditions for the practical introduction of vasopressin antagonists is that they should be highly potent and selective compounds. The action of the present antidiuretic antagonist has also been described by other authors [8, 9, 11]. This compound appears to be of promise from the aspect of clinical application. However, two problems must be considered: (a) The strength of the effect of the antagonist is much lower than that of the naturally occurring hormone agonist, arginine vasopressin. In human [3] and in rat [2, 10] the effective circulating hormone level is in the pg/ml order, whereas the antidiuretic effect of endogenous vasopressin could be blocked with a 30 $\mu$g/kg dose. The dose difference is even more striking in the experiment aimed at averting the antidiuresis caused by exogenous vasopressin. To halve the antidiuretic effect of 10 pg (30 $\mu$U) arginine vasopressin, 4.0—4.3 $\mu$g antagonist had to be administered to the homozygous Brattleboro rats, with no endogenous vasopressin reserve. (b) $d(CH_2)_5$Tyr(Et)VAVP possesses an appreciable antipressor effect. The "effective antipressor dose" of the compound (0.49 nmol/kg = 0.56 $\mu$g/kg) is lower than the effective antidiuretic dose. When the drug is used, therefore, the blood pressure-decreasing effect must be taken into account.

Recently, D-tyrosine has been built into the molecule in place of the L-tyrosine at position 2 [9]. The "effective dose" of the antidiuretic antagonist $d(CH_2)_5$-D-Tyr(Et)VAVP is 1.1 nmol/kg, but the strength of its antipressor effect is the same as that of the L-tyrosine analogue. The most potent antidiuretic antagonist compound found so far is $d(CH_2)_5$-D-Phe-VAVP ("effective dose" = 0.67 nmol/kg); here too the antipressor action is marked ("effective dose" = 0.58 nmol/kg) [7]. The most promising compound appears to be $d(CH_2)_5$-D-Leu-VAVP, which primarily exerts antidiuretic antagonist action ("effective dose" = 1.2 nmol/kg) [7], while its antipressor effect is more than 20 times weaker ("effective dose" = 26 nmol/kg).

In human therapy the compound seems to be of promise primarily in the treatment of the Schwartz—Bartter syndrome. In our experiments we attempted to induce in rat conditions corresponding very closely to those in the human

syndrome. In the event of sufficient hydration, marked water retention, hypernatriuria, hyponatraemia and cerebral oedema could be induced by administration of a large dose of a long-acting vasopressin preparation (Pitressin tannate). These changes could be prevented by the simultaneous administration of $d(CH_2)_5Tyr(Et)VAVP$. Our observations support the hypothesis that this compound might be of use in the future as an effective drug in the treatment of the Schwartz—Bartter syndrome.

## REFERENCES


1. De Wied, D.: A simple automatic and sensitive method for the assay of antidiuretic hormone with notes on the antidiuretic potency of plasma under different experimental conditions. Acta Physiol. Pharmacol. Neerl. **9,** 69—81 (1960)
2. Dogterom, J., van Wimersma Greidanus, Tj., de Wied, D.: Vasopressin in cerebrospinal fluid and plasma of man, dog and rat (Wistar and Brattleboro) Amer. J. Physiol. **234,** 463—467 (1978)
3. Edwards, C. R. W.: Vasopressin. In: Clinical Endocrinology, pp 527—567 Eds.: Martini, L., Besser, G. M., Academic Press, New York, 1977
4. Kovács, K., László, F. A., Dávid, M. A.: The antidiuretic phase of water metabolism in rats after lesions of the pituitary stalk. I. J. Endocrin. **25,** 387—396 (1962)
5. László, F. A., Kovács, K.: Role of pituitary stalk in water metabolism and regulation of ACTH-adrenocortical system. pp. 27—39. Studia Medica, Szeged, 1968
6. Manning, M., Grzonka, Z. Sawyer W. H.: Synthesis of posterior pituitary hormones and hormone analogues. In: "The Pituitary". Eds.: Beardwell, C., Robinson, E. G. pp. 265—296. Butterworths, Kent England, 1981
7. Manning, M., Klis, W. A., Olma, A., Seto, J. Sawyer, W. H.: Design of More Potent and Selective Antagonists of the Antidiuretic Responses to Arginine-vasopressin Devoid of Antidiuretic Agonism. J. Med. Chem. **25,** 414—419 (1982)
8. Manning, M., Lammek, B., Kolodziejczyk, A. M., Seto, J. Sawyer, W. H.: Synthetic Antagonists of in Vivo Antidiuretic and Vasopressor Response to Arginine-vasopressin. J. Med. Chem. **24,** 701—706 (1981)
9. Manning, M., Olma, A., Klis, W. A., Kolodziejczyk, A. M., Seto, J. Sawyer, W. H.: Design of More Potent Antagonists of the Antidiuretic Responses to Arginine-Vasopressin. J. Med. Chem. **25,** 45—50 (1982)
10. Moore, G., Lutterodt, A., Burford, G., Lederis, K.: Highly specific antiserum for arginine vasopressin. Endocrinology **101,** 1421—1435 (1977)
11. Sawyer, W. H., Pang, P. K. T., Seto, J., McEncore, M., Lammek, B. Manning, M.: Vasopressin Analogs that Antagonize Antidiuretic Responses by Rats to the Antidiuretic Hormone. Science, **212,** 49—51 (1981)
12. Schwartz, W. B., Bennett, W., Curelop, S., Bartter, F. C.: A syndrome of renal sodium loss and hyponatremia, probably resulting from inappropriate secretion of antidiuretic hormone. Amer. J. Med. **23,** 529—542 (1957)

PRINTED IN HUNGARY
Akadémiai Kiadó és Nyomda, Budapest

# INFORMATION FOR AUTHORS

*Acta Medica Hungarica* is published under the auspices of the Hungarian Academy of Sciences. Manuscripts and editorial correspondence should be sent to the editorial office: H-1450 Budapest 9, P. O. Box 67.

Original articles dealing with clinical and experimental medicine will be accepted with the understanding that they have not been and will not be published elsewhere and are subject to editorial revision.

*Form of manuscripts*

Two copies of the manuscript typewritten double-spaced with margins at least 4 cm wide should be submitted. Pages should be numbered consecutively. The first page should contain (1) the title of the paper (2) the initials and first name(s) of the author(s), (3) name of the institution where the work was done, (4) name and address of the author to whom correspondence and offprint requests should be addressed — this will appear as a footnote; (5) an abstract not exceeding 250 works which states the purposes of the study, the main findings and principal conclusions. Below the abstract provide 3 to 10 keywords that will assist indexers in cross-indexing the article.

The text of the paper should be divided into sections with the headings: Introduction, Materials (Patients) and Methods, Results, Discussion, References.

Unusual abbreviations should be identified in an alphabetical list typed after the abstract and keywords.

Drugs must be referred to by their WHO code designation (Recommended International Nonproprietary Names); use of proprietary names is unacceptable.

The international system of units (SI) should be used for all measurements.

*References*

These should be cited in the text as numbers in square brackets. The list of references should contain in alphabetical order of the first authors' names the following: authors' last names with initials; for journal articles the title of the paper (lower case), journal title abbreviated according to the style used in Index Medicus, volume number, inclusive page numbers, year of publication in parentheses; for books the title (upper and lower case), publisher, place and date of publication. Only manuscripts accepted for publication may be included in the reference list.

Examples:

1. Stagg, B. H., Temperly, J. M., Wyllie, J. H.: The fate of pentagastrin. Gut **12**, 825—829 (1971)
2. Falkner, F.: Prevention in Childhood of Health Problems and Adult Life. WHO, Geneva 1980.
3. Fishman, A. P.: Dynamics of pulmonary circulation. In: Hamilton, W. F., Dow, P. (eds): Handbook of Physiology. American Physiological Society, Washington 1963, pp. 65—79.

*Tables*

Each table should be typed on a separate sheet. They should be numbered consecutively with Roman numerals and have a brief specific title. The data presented in the table must be logically and clearly organized and should be self-explanatory. Omit internal horizontal and vertical rules. Cite each table in the text and indicate its approximate place on the margin.

*Illustrations*

Figures should be submitted in duplicate. They must be numbered consecutively with arabic numerals. All figures should bear the name of the first author, the figure number and an arrow indicating the top. Cite each figure in the text and indicate its approximate place on the margin. If a figure has been published, acknowledge the original source and submit written permission from the copyright holder to reproduce the material. Figure captions should be submitted typed double-spaced on a separate sheet.

*Proofs and reprints*

The first authors will receive (1) comments and suggestions of the Editorial Board for improving their paper; (2) a set of proofs for correction; corrected proofs should be returned without delay to the editorial office; (3) 100 reprints free of charge.

## Periodicals of the Hungarian Academy of Sciences are obtainable at the following addresses:

**AUSTRALIA**
C.B.D. LIBRARY AND SUBSCRIPTION SERVICE
Box 4886, G.P.O., *Sydney N.S.W. 2001*
COSMOS BOOKSHOP, 145 Ackland Street
*St. Kilda (Melbourne), Victoria 3182*

**AUSTRIA**
GLOBUS, Höchstädtplatz 3, *1206 Wien XX*

**BELGIUM**
OFFICE INTERNATIONAL DE LIBRAIRIE
30 Avenue Marnix, *1050 Bruxelles*
LIBRAIRIE DU MONDE ENTIER
162 rue du Midi, *1000 Bruxelles*

**BULGARIA**
HEMUS, Bulvar Ruszki 6, *Sofia*

**CANADA**
PANNONIA BOOKS, P.O. Box 1017
Postal Station "B", *Toronto, Ontario M5T 2T8*

**CHINA**
CNPICOR, Periodical Department, P.O. Box 50
*Peking*

**CZECHOSLOVAKIA**
MAD'ARSKÁ KULTURA, Národní třida 22
*115 66 Praha*
PNS DOVOZ TISKU, Vinohradská 46, *Praha 2*
PNS DOVOZ TLAČE, *Bratislava 2*

**DENMARK**
EJNAR MUNKSGAARD, Norregade 6
*1165 Copenhagen K*

**FEDERAL REPUBLIC OF GERMANY**
KUNST UND WISSEN ERICH BIEBER
Postfach 46, *7000 Stuttgart 1*

**FINLAND**
AKATEEMINEN KIRJAKAUPPA, P.O. Box 128
*SF-00101 Helsinki 10*

**FRANCE**
DAWSON-FRANCE S. A., B. P. 40, *91121 Palaiseau*
EUROPÉRIODIQUES S. A., 31 Avenue de Versailles, *78170 La Celle St. Cloud*
OFFICE INTERNATIONAL DE DOCUMENTATION ET LIBRAIRIE, 48 rue Gay-Lussac
*75240 Paris Cedex 05*

**GERMAN DEMOCRATIC REPUBLIC**
HAUS DER UNGARISCHEN KULTUR
Karl Liebknecht-Straße 9, *DDR-102 Berlin*
DEUTSCHE POST ZEITUNGSVERTRIEBSAMT
Sraße der Pariser Kommüne 3–4, *DDR-104 Berlin*

**GREAT BRITAIN**
BLACKWELL'S PERIODICALS DIVISION
Hythe Bridge Street, *Oxford OX1 2ET*
BUMPUS, HALDANE AND MAXWELL LTD.
Cowper Works, *Olney, Bucks MK46 4BN*
COLLET'S HOLDINGS LTD., Denington Estate
*Wellingborough, Northants NN8 2QT*
WM. DAWSON AND SONS LTD., Cannon House
*Folkstone, Kent CT19 5EE*
H. K. LEWIS AND CO., 136 Gower Street
*London WC1E 6BS*

**GREECE**
KOSTARAKIS BROTHERS INTERNATIONAL BOOKSELLERS, 2 Hippokratous Street, *Athens-143*

**HOLLAND**
MEULENHOFF-BRUNA B.V., Beulingstraat 2,
*Amsterdam*
MARTINUS NIJHOFF B.V.
Lange Voorhout 9–11, *Den Haag*
SWETS SUBSCRIPTION SERVICE
347b Heereweg, *Lisse*

**INDIA**
ALLIED PUBLISHING PRIVATE LTD., 13/14
Asaf Ali Road, *New Delhi 110001*
150 B-6 Mount Road, *Madras 600002*
INTERNATIONAL BOOK HOUSE PVT. LTD.
Madame Cama Road, *Bombay 400039*
THE STATE TRADING CORPORATION OF INDIA LTD., Books Import Division, Chandralok
36 Janpath, *New Delhi 110001*

**ITALY**
INTERSCIENTIA, Via Mazzé 28, *10149 Torino*
LIBRERIA COMMISSIONARIA SANSONI, Via Lamarmora 45, *50121 Firenze*
SANTO VANASIA, Via M. Macchi 58
*20124 Milano*
D. E. A., Via Lima 28, *00198 Roma*

**JAPAN**
KINOKUNIYA BOOK-STORE CO. LTD.
17-7 Shinjuku 3 chome, Shinjuku-ku, *Tokyo 160-91*
MARUZEN COMPANY LTD., Book Department, P.O. Box 5050 Tokyo International, *Tokyo 100-31*
NAUKA LTD. IMPORT DEPARTMENT
2-30-19 Minami Ikebukuro, Toshima-ku, *Tokyo 171*

**KOREA**
CHULPANMUL, *Phenjan*

**NORWAY**
TANUM-TIDSKRIFT-SENTRALEN A.S., Karl Johansgatan 41–43, *1000 Oslo*

**POLAND**
WĘGIERSKI INSTYTUT KULTURY, Marszałkowska 80, *00-517 Warszawa*
CKP I W, ul. Towarowa 28, *00-958 Warszawa*

**ROUMANIA**
D. E. P., *Bucureşti*
ILEXIM, Calea Grivitei 64–66, *Bucureşti*

**SOVIET UNION**
SOJUZPECHAT – IMPORT, *Moscow*
and the post offices in each town
MEZHDUNARODNAYA KNIGA, *Moscow G-200*

**SPAIN**
DIAZ DE SANTOS, Lagasca 95, *Madrid 6*

**SWEDEN**
ALMQVIST AND WIKSELL, Gamla Brogatan 26
*101 20 Stockholm*
GUMPERTS UNIVERSITETSBOKHANDEL AB
Box 346, *401 25 Göteborg 1*

**SWITZERLAND**
KARGER LIBRI AG, Petersgraben 31, *4011 Basel*

**USA**
EBSCO SUBSCRIPTION SERVICES
P.O. Box 1943, *Birmingham, Alabama 35201*
F. W. FAXON COMPANY, INC.
15 Southwest Park, *Westwood Mass. 02090*
THE MOORE-COTTRELL SUBSCRIPTION AGENCIES, *North Cohocton, N. Y. 14868*
READ-MORE PUBLICATIONS, INC.
140 Cedar Street, *New York, N. Y. 10006*
STECHERT-MACMILLAN, INC.
7250 Westfield Avenue, *Pennsauken N. J. 08110*

**YUGOSLAVIA**
JUGOSLOVENSKA KNJIGA, Terazije 27, *Beograd*
FORUM, Vojvode Mišića 1, *21000 Novi Sad*

Index: 26.015

# Acta Medica Hungarica

VOLUME 41, NUMBERS 2—3, 1984



Akadémiai Kiadó, Budapest
ACTA MED. HUNG. 41(2-3) 77—173 (1984) HU ISSN 0236-5286

# ACTA MEDICA HUNGARICA

A QUARTERLY OF THE HUNGARIAN ACADEMY OF SCIENCES

*Acta Medica* publishes reviews and original papers on clinical and experimental medicine in English.

*Acta Medica* is published in yearly volumes of four issues by

AKADÉMIAI KIADÓ

Publishing House of the Hungarian Academy of Sciences
H-1054 Budapest, Alkotmány u. 21

Manuscripts and editorial correspondence should be addressed to the Managing Editor:

Dr. Miklós Papp
*Acta Medica*
H-1083 Budapest, Szigony u 43 or H-1450 Budapest 9, P. O. Box 67

*Subscription information*

Orders should be addressed to

KULTURA Foreign Trading Company
H-1389 Budapest, P. O. Box 149

*Acta Medica is indexed in Current Contents*

CONTENTS

*Acta Medica Hungarica, 41 (2—3), pp. 77—92 (1984)*

*Psychiatry – Mutagenicity*

# SELF-POISONING AS A MODEL FOR THE STUDY OF THE MUTAGENICITY OF CHEMICALS IN HUMAN BEINGS

A. Czeizel, I. Szentesi, G. Molnár

DEPARTMENT OF HUMAN GENETICS, NATIONAL INSTITUTE OF HYGIENE, AND DEPARTMENT OF TOXICOLOGICAL MEDICINE, S. AND F. KORÁNYI HOSPITAL, BUDAPEST, HUNGARY

Received: February 11, 1983

Individuals who poison themselves in a suicide attempt with extremely large doses of chemicals may comprise a population that is useful for the study of the possible mutagenicity of drugs and other compounds. After the demonstration of the demographic data of completed and attempted suicide cases, the methodology of short-term somatic and long-term germinal mutagenesis systems are described. Finally the advantages and disadvantages of this human experimental epidemiological model are discussed, pointing out the practical and theoretical importance of the approach.

**Keywords:** self-poisoning, suicide attempt, mutagenicity of drugs, human experimental model

## Introduction

It would be best if human beings as a test system could be excluded from research. The fulfilment of this postulate is particularly true in medical genetics and within it, in dysgenics i.e. the study of mutagenesis. Thus it would be important to identify potential mutagens with sensitive and reliable screening systems of lower organisms and to prevent their contact with the human population. We are, however, far from this ideal case. First, the screening systems are imperfect and are not enough widespread in use. Secondly, owing to species differences, the extrapolation of results from experimental animals to human beings can be realized with difficulties. Thirdly, mankind is permanently exposed to many naturally occurring chemicals. "Consequently, the harsh reality is that observations on human beings will provide the ultimate information" [9]. Thus man himself as a unique authentic measure could not be excluded from the study of mutagenesis and it is important to develop efficient epidemiological methods for human populations. The subject of this study will be the populations at risk exposed to one or more specific compounds.

Send offprint requests to A. Czeizel, National Institute of Hygiene, H-1966 Budapest, Gyáli út 2–6

In recent years there has been an increased interest in the studies of chronic low doses of potential mutagens, e.g. occupational and medical exposures [3]. In contrast, there are few studies of large doses of chemicals e.g. in the victims of accidents. Still, as the first publication of ICPEMC [2] entitled "Genetic monitoring of human populations accidentally exposed to a suspected mutagenic chemical" stated: "There is currently little relevant information on mutagenesis which can be directly applied to humans because the most readily observable effects may be relatively rare and it is often difficult to locate groups which have been exposed to one specific factor . . . For this reason close study of the victims of accidental exposures could yield unique scientific information which would provide the data base for future application. We therefore strongly advocate that the groups of accidentally exposed

**Table I**

*Death rates from suicide in some selected countries on 1979*

| Selected countries | Total deaths No. | Suicide and self-inflicted injury | |
|---|---|---|---|
| | | No. | per 100 000 persons |
| Europe | | | |
| Austria | 92 012 | 1 883 | 25.1 |
| Bulgaria | 94 403 | 1 267 | 14.4 |
| Denmark | 54 369 | 1 318 | 25.8 |
| England | 593 019 | 4 195 | 8.5 |
| Germany F.R.* | 723 218 | 13 620 | 22.2 |
| Greece* | 81 615 | 290 | 2.9 |
| Hungary | 136 829 | 4 770 | 44.6 |
| Italy*** | 550 565 | 3 199 | 5.7 |
| Netherlands | 112 565 | 1 465 | 10.4 |
| Norway | 41 632 | 494 | 12.1 |
| Poland* | 325 104 | 4 640 | 13.3 |
| Spain** | 294 324 | 1 486 | 4.1 |
| Sweden | 91 060 | 1 703 | 20.5 |
| Switzerland | 57 454 | 156 | 24.7 |
| Other continents | | | |
| USA* | 1 927 788 | 27 294 | 12.5 |
| Canada** | 167 498 | 3 317 | 14.2 |
| Japan | 689 664 | 20 823 | 18.0 |
| Israel* | 25 153 | 208 | 5.6 |

* Data from 1978
** Data from 1977
*** Data from 1970

people be studied thoroughly . . . " Besides the victims of accidents, the subjects who commit suicide by taking chemicals may represent similar — and perhaps even more adequate — material for the study of mutagenicity of large doses of chemical compounds. The features and utility of this human model will be discussed in this paper but first an attempt is made to characterize the data derived from such individuals.

*Completed suicide*

Table I explains the particular interest of Hungarian experts in the death rate from suicide. According to statistical data, Hungary has an extraordinary high death rate caused by suicide (so-called completed suicide) and self-inflicted injury. Some other European countries such as Austria, Denmark, Finland and Switzerland have about 25 prevalences. Previously high values were known from East-Germany (e.g. 34.0 in 1974) but recently these figures have not been published. Europe has a peculiar distribution of suicide mortality (Fig. 1). Hungary is the epicentrum, in general the central-European countries have intermediate values, while countries in the Mediterranean area as well as the U.K. and the Netherlands show low prevalences.

The highest Hungarian value has been the consequence of a continuously increasing trend from 1951, though it has been high since the last decades of the last century (Fig. 2). In Hungary the suicide deaths seem to be endemic

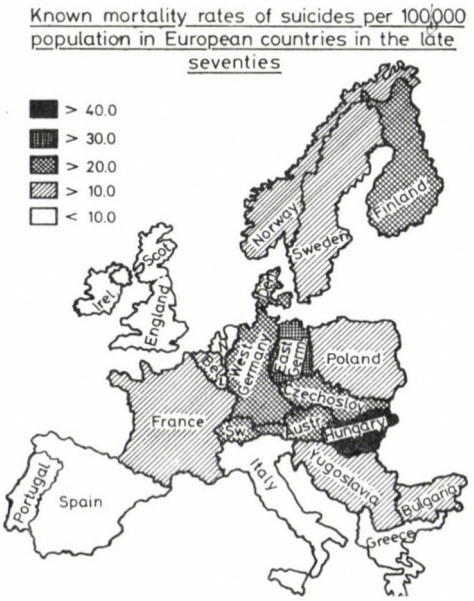



*Fig. 1*

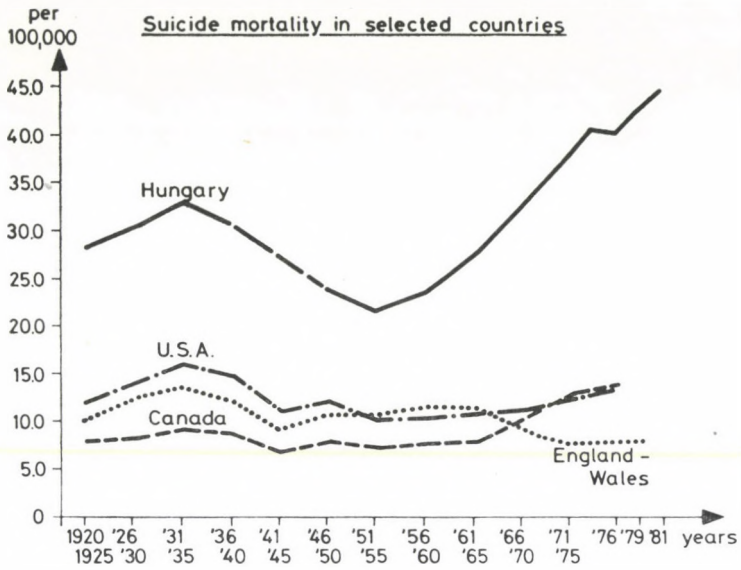



*Fig. 2*

i.e. habitually prevalent and due to permanent local causes. What are these causes? An important point may be the high proportion of violent methods. The efficacy of suicide methods is considerably different, and the distribution of methods is not the same in various countries. The Hungarian percentage rates of suicide methods are shown in Table II. (The Hungarian ones originate from the years 1965–1974 because reliable figures are available from that period.) The predominance of hanging with high efficacy is obvious in Hungary. The other explanations of the very high Hungarian values may be the more reliable statistical registration, the increasing rate of old people and some traditional social patterns. The point is that suicide is among the main causes of deaths in Hungary (Table III).

From the genetic point of view, a completed suicide does not pose a problem, because it is a kind of man-made biological selection, and the possibility of late consequences can be excluded. The long-term mutagenic and carcinogenic effects, however, may be important in the later life of attempted suicide cases and probably in their offspring. Thus, the data of such persons deserve particular attention.

### *Attempted suicide with chemicals*

Self-poisoning and attempted suicide are not identical terms [10, 19], but in the majority of our cases, self-poisonings were attempted suicides, thus these terms will be used as synonyms in this paper.

**Table II**

*Percentage of methods for persons who had committed or attempted suicide in Hungary, 1964–1975*

| Method | Completed suicide | | | Attempted suicide | | | Efficacy |
|---|---|---|---|---|---|---|---|
| | male | female | total | male | female | total | |
| Self-poisoning | | | | | | | |
| drugs | 8.4 | 26.7 | 13.9 | 73.0 | 89.0 | 84.1 | 4.3 |
| gas | 3.0 | 8.1 | 4.5 | 2.3 | 1.8 | 2.0 | 41.1 |
| other substances | 9.9 | 10.8 | 10.1 | 7.8 | 4.0 | 5.2 | 34.7 |
| Stabbing | 1.9 | 1.2 | 1.7 | 8.1 | 2.6 | 4.1 | 9.9 |
| Hanging | 61.6 | 35.1 | 53.7 | 4.4 | 0.6 | 1.8 | 88.9 |
| Drowning | 3.7 | 9.0 | 5.3 | 1.0 | 0.6 | 0.7 | 67.4 |
| Leaping | 2.5 | 4.6 | 3.1 | 0.4 | 0.2 | 0.3 | 73.4 |
| Running over | 4.0 | 3.5 | 3.9 | 0.7 | 0.2 | 0.4 | 76.0 |
| Fire-arms | 3.6 | 0.2 | 2.6 | 0.4 | 0.0 | 0.1 | 84.2 |
| Electric shock | 1.2 | 0.7 | 1.1 | 0.9 | 0.3 | 0.5 | 42.5 |
| Others | 0.2 | 0.1 | 0.1 | 1.0 | 0.7 | 0.8 | 33.5 |
| Total | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 21.4 |

**Table III**

*Main causes of deaths in Hungary, 1978*

| Causes of death | Per cent |
|---|---|
| Ischaemic heart disease | 20.8 |
| Neoplasm | 19.2 |
| Cerebrovascular disease | 14.3 |
| Accident | 5.3 |
| Hypertensive disease | 4.8 |
| Bronchitis, emphysema, asthma | 3.5 |
| Other forms of heart disease | 3.4 |
| Suicide and self-inflicted injury | 3.3 |
| Cirrhosis of liver | 1.7 |
| Others | 23.7 |
| Total | 100.0 |

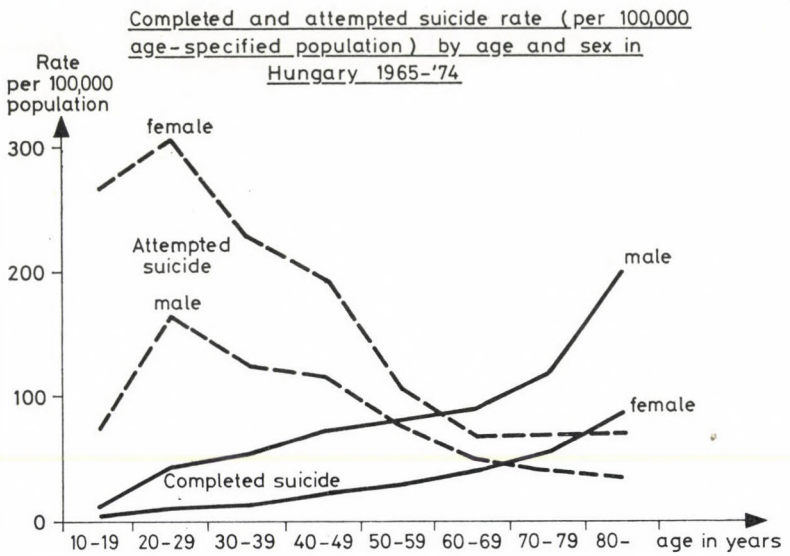



*Fig. 3*

Both the sex ratio and the age distribution of suicide attempters differ significantly from the ratios of completed suicide (Fig. 3). While there is a significant male preponderance in completed suicides (70% in Hungary), only 32% of the attempted suicides are male. The age specific incidences are also reversed i.e. the completed suicide death rates increase with advancing age in both sexes. In contrast, the majority of suicide attempters is found among young people of reproductive age. The age distribution of fertility has a somewhat similar pattern, but the peak of suicide attempts (at the age of 17–19) precedes the peak of childbirths (at the age of 21–26).

Drugs and other chemical substances account for 90% of attempted suicides. In Great Britain there has been a 20-fold increase in the annual number of admissions to hospital in the last 20 years due to what has been called an "epidemic of self-poisoning" [8]. It amounted to almost 10% of all admissions and 20% of all medical emergencies was self-poisoning in Sheffield in the early 1970's [22]. In some areas poisonings are a major cause of admission to hospital, more than ischaemic heart disease [8]. The efficacy of self-poisoning by drugs is low and the mortality shows a decreasing trend: only 2.6% of persons who had attempted suicide by drugs died in Budapest in 1979.

The ratio of completed to attempted suicides ("parasuicides") shows a changing pattern in different countries. The estimated ratios are 1 to 7–8 [21] and 1 to 39 [13], respectively, in the USA and 1 to 9.7 in England and Wales [14], while in Hungary the ratio of completed suicides to registered attempted

suicides is 1 to 3.7. Naturally, patients who did not seek medical advice after a suicidal attempt or those who were treated at home by general practitioners are not included in these figures. They may be a certain part of suicide attempts mainly with weak poisonings. Furthermore some country hospitals do not report all attempted suicides. The real ratio might be around 1 to 5–8 in Hungary.

According to calculations made by Cseh-Szombathy and Czeizel (Table IV), based on the registered figures of attempted suicide in the 15–34 year

**Table IV**

*Estimation of rate of attempted suicides between 15 and 34 years of age Hungary, 1965–1974*

| Estimation \ Age group | 15-19 | 20-24 | 25-29 | 30-34 | Total |
|---|---|---|---|---|---|
| Registered per cent of attempted suicides | 0.29 | 0.25 | 0.20 | 0.17 | 0.23 |
| Corrected per cent of attempted suicides | 0.58 | 0.50 | 0.40 | 0.34 | 0.46 |
| Cumulated per cent of age-group cohort | 2.90 | 2.50 | 2.00 | 1.70 | 2.30 |
| Cumulated per cent of total cohort | | | 9.10 | | |

age-groups, the range was 0.17–0.29% with a mean value of 0.23%. Owing to the obvious underestimation of self-poisoning cases a correction was necessary. The corrected value is 0.46%, a two-fold increase. The cumulated per cent of the age group cohort of attempted suicides ranged from 1.7 to 2.9% and that of the total cohort was 9.1%. (Our figures were limited only to these age-groups, because in Hungary more than 95% of the mothers deliver their babies before the age of 34.) Since this 9.1% figure may seem incredibly high, some comments are necessary. (*i*) In 1972 an estimated annual suicide attempt rate of 0.38% was obtained for Boston [13] thus there the cumulated total cohort value may be even higher, (*ii*) the 9.1% figure is in keeping with the 3.3% of all deaths caused by suicide and self-inflicted injury in Hungary, 1978 (Table III), and (*iii*) the per cent of repeaters has to be deducted from this 9.1%. The registered repeat rate is 12.9% and the true rate is estimated at double. Thus the per cent of the cumulated total cohort value of suicide attempters is 6.8%. Since 90% of the suicide attempters use drugs, the rate of self-poisoning persons within the 15–34 year age-group may be about 6%, i.e. 1 in 17 persons.

The main conclusion is that self-poisoning by chemicals is a common social problem, so that mutagenic effect of attempted suicide must also be taken into consideration both in the later life of suicide attempters as well as

in their offspring. The incidence of these cases is far greater than of the victims of accidents and of patients treated with cytostatics under the age of 34 [12, 24].

Summing up the data of suicide attempters from the genetic point of view, we have to face some threatening facts:

1. The number of suicide attempters is very high.

2. Within the attempted suicides the percentage of self-poisoning shows a dramatic rise and it is one of the major medicosocial phenomena of the past 30 years.

3. It is paradoxical to say that because of a more and more effective medical treatment the ratio of survivors with possible long-term consequences increases continuously.

4. One of the most threatening points is the predominance of young among the suicide attempters people who may have children later.

5. The new pattern of suicide morbidity shows that the majority of suicide attempters are not physically ill, thus previously healthy organisms are subjected to self-poisoning [16].

The mutagenic monitoring of self-poisoning persons seems to be necessary for two reasons. First as a high risk population, and secondly as a model for the study of the mutagenic effect on human beings of large doses of different chemicals.

*Study of the short-term somatic mutagenic effect*

Since 1971 we have systematically studied the somatic mutations of persons who had attempted suicide by chemicals [1, 4, 11, 23].

This approach involves the study of short-term effects of acute poisoning in persons attempting suicide by drugs. The first and second blood samples were taken at 4–8 h (Study I) and at about the 72nd h (Study II) after the poisoning. Chromosome aberrations including the usual study of breakage analysis of chromatid and chromosome type aberrations and the study of stable chromosome aberrations with G-banding technique, sister chromatid exchanges (SCEs) and some immunoproteins (IgA, IgC, IgM and C3) were analysed in peripheral blood samples. The third blood sample (Study III) was planned to be taken at about three months after poisoning and the above-mentioned methods were completed by analysis of sperm abnormalities and menstrual disorders.

A sample of individuals from the surgical department of the same hospital matched for sex, age, and socio-economic conditions with the exposed persons, ensuring a closely similar environmental background, served as the control. These individuals underwent surgery for appendicitis or hernia.

The blood samples from both the control and study II of the exposed groups were processed together. Bias in scoring was avoided by coding the samples and the scorer had no prior knowledge of which slides were from exposed and which from control samples. Chromosome aberrations were evaluated after approximately 72 h of cultivation at 37°C. It is well-known that for the study of chromosome aberrations in lymphocytes following in vivo exposure of individuals to suspected mutagens, the best culturing time is 50 h [7]. A suitable number of mitoses did not, however, appear in 50 h culture from acutely poisoned persons, so a longer culturing was sometimes necessary.

The method for the detection of SCEs was essentially the same as reported earlier by Raposa [18] and Perry and Wolff [17]. IgA, IgG, IgM and Complement Component C3 were studied by the Beckman Immunochemistry System. The DNA-repair capacity and HGPRT locus testing have also been studied.

The chromosome aberrations and sister chromatid exchanges showed a transient increase after the poisoning [3], in the first part of our study. At present, in the second part of our research we deal with the evaluation of mutagenic effect of separating chemicals.

*Study of the long-term germinal mutagenic effect*

In 1979–1981 an epidemiological follow-up cohort study was organized to analyse pregnancy outcomes of individuals who previously had poisoned themselves. The specific question was whether in semilethally intoxicated persons large doses of chemicals produced germinal mutations with epidemiologically detectable consequences in their subsequent reproductive outcome. The answer to this question was negative as there was no evidence whatever of germinal mutations produced by epidemiologically detectable semilethal drug poisoning [6]. Here the questionnaire used for personal interview is shown (Table V) and the distributions of the so-called confounding variables are summarized in the study sample 1399 persons (*i*) attempting suicide (*ii*) with extremely large doses of chemicals causing of unconsciousness for at least one day (*iii*) below the age of 30 years (guaranteeing subsequent reproductive activity), (*iv*) treated in 1960–1967 (allowing time for realisation of the reproductive potential), (*v*) in a special department of poisoned persons in Budapest and in the control sample 881 persons (*i*) who had surgery for varicosity, appendicitis and hernia repair (*ii*) in the surgical department of the same hospital (*iii*) in 1960–1967 (*iv*) matched for sex, age, and district of residence. All male index cases were matched but owing to the preponderance of females among the suicide attempters, age and district concordant pairs were selected from female index cases and each pair had only one matched control female. Nearly, 7 400 control cases were obtained

**Table V**

*Data of questionnaire*

Questionnaire for family planning attitude

A) *Personal data:*
1. Data of birth
2. Present marital status
3. Previous marital status (at time of self-poisoning)
4. Highest educational qualification

B) *Health status*
5. Occupation, working places (period), possible occupational exposition and risk (name)
6. Main diseases: date (duration) and names
7. Hospitalization: date (duration) and cause
8. Chronic drug treatment under physician guidance: date (duration) and names of drugs
9. Diagnostic and therapeutic X-ray or isotope: date and cause
10. Acute poisoning by chemicals or drugs; date and name
11. Smoking: duration and quantity per day
12. Alcoholic drinks: duration and quantity per day/per week

C) *Obstetric events*
I. Number of livebirths. Date of birth, Sex, Birth weight.
II. Number of stillbirths. Date of birth, Sex, Abnormality in fetus.
III. Number of spontaneous abortions. Date of events, Sex, Cause.
IV. Number of ectopic pregnancies. Date of events
V. Number of induced abortions. Date of events, Cause
VI. Any events during pregnancy (date, name, consequences)
VII. Occurrence of delayed conception and/or infertility (cause)
VIII. In cases of planned pregnancy how many cycles did it take to become pregnant

D) *Main diseases (and cause of death if died) of children*
Anomaly, Repeated infections, Tumour, Hospitalization (name, time)
Other

Remarks

*Name of affected children*
Address (if not mother's home address)
Name and address of the physician
Other remarks
Date Name of questioning official

from the medical files, thus all index cases were matched from the reserve material if the original cases could not be found. Unpaired control cases were excluded.

## *Confounding factors*

There was a highly significant difference in marital status between the males in the study and the control sample at the time of attempted suicide (Table VI): 3.5 times more males were married among the self-poisoning persons. In females the difference was much lower.

Distribution of the educational level according to the finished school grade did not differ in the study and control samples (Table VII). Nevertheless, mainly in the matched control males there was a predominance of higher grades.

**Table VI**

*Marital status of self-poisoning and matched control cases at the time of self-poisoning (SP) and of the epidemiological study (ES)*

| Sample | Sex | Time | Married | | Unmarried | | Total | |
|---|---|---|---|---|---|---|---|---|
| | | | No. | per cent | No. | per cent | No. | per cent |
| Self-poisoning persons | Male | SP | 205 | 56.5 | 158 | 43.5 | 363 | 100.0 |
| | | ES | 219 | 60.3 | 144 | 39.7 | 363 | 100.0 |
| | Female | SP | 202 | 19.5 | 834 | 80.5 | 1036 | 100.0 |
| | | ES | 691 | 66.7 | 345 | 33.3 | 1036 | 100.0 |
| Matched control cases | Male | SP | 59 | 16.3 | 304 | 83.7 | 363 | 100.0 |
| | | ES | 199 | 54.8 | 164 | 45.2 | 363 | 100.0 |
| | Female | SP | 54 | 10.4 | 464 | 89.6 | 518 | 110.0 |
| | | ES | 311 | 60.0 | 207 | 40.0 | 518 | 100.0 |

**Table VII**

*Distribution of educational level in the study and control samples*

| Number of school grades \ Sample | Self-poisoning | | | | Matched control | | | |
|---|---|---|---|---|---|---|---|---|
| | Male | | Female | | Male | | Female | |
| | No. | per cent | No. | per cent | No. | per cent | No. | per cent |
| 0—7 | 18 | 5.0 | 24 | 2.3 | 19 | 5.2 | 24 | 4.6 |
| 8 | 133 | 36.6 | 426 | 41.1 | 112 | 30.9 | 215 | 41.5 |
| 9—11 | 107 | 29.5 | 145 | 14.0 | 106 | 29.2 | 53 | 10.2 |
| 12 | 76 | 20.9 | 373 | 36.0 | 89 | 24.5 | 187 | 36.1 |
| 13—18 | 29 | 8.0 | 68 | 6.6 | 37 | 10.2 | 39 | 7.6 |
| Total | 363 | 100.0 | 1036 | 100.0 | 363 | 100.0 | 518 | 100.0 |

Evaluation of the occupational distribution showed an opposite trend in self-poisoning males and females (Table VIII). In self-poisoning males there was significant surplus of occupations with lower qualification (semi- and unskilled) or without employment (mainly owing to ill-health). They did not correspond to their educational distribution, showing a social come-down. In self-poisoning females the skilled workers and persons without employment were predominating and the per cent of semi- and unskilled workers was lower

**Table VIII**

*Occupational distribution of index and control cases*

| Occupation \ Sample | Self-poisoning | | | | Matched control | | | |
|---|---|---|---|---|---|---|---|---|
| | Male | | Female | | Male | | Female | |
| | No. | per cent | No. | per cent | No. | per cent | No. | per cent |
| Intellectual | 28 | 7.7 | 59 | 5.7 | 41 | 11.3 | 46 | 8.9 |
| Clerk | 8 | 2.2 | 67 | 6.5 | 5 | 1.4 | 29 | 5.6 |
| Skilled worker | 166 | 45.7 | 337 | 32.5 | 231 | 63.7 | 115 | 22.2 |
| Semi-skilled worker | 97 | 26.7 | 354 | 34.2 | 56 | 15.4 | 224 | 43.2 |
| Unskilled worker | 40 | 11.0 | 135 | 13.0 | 15 | 4.1 | 81 | 15.7 |
| Unemployed | 24 | 6.7 | 84 | 8.1 | 15 | 4.1 | 23 | 4.4 |
| Total | 363 | 100.0 | 1036 | 100.0 | 363 | 100.0 | 518 | 100.0 |

than that in the matched control sample. The rate of possible dangerous occupational exposures of self-poisoning persons did not differ from their matched control cases.

The per cent of habitual drinkers was significantly higher in both sexes of the study sample than in the control group (Table IX).

**Table IX**

*Estimated consumption of alcoholic beverages in the self-poisoning and matched control persons at the time of the epidemiological study*

| Degree of drinking | | Self-poisoning persons | | | Matched control | | |
|---|---|---|---|---|---|---|---|
| | | Male | Female | Together | Male | Female | Together |
| Habitual | No. | 84 | 65 | 149 | 16 | 9 | 25 |
| | Per cent | 23.1 | 6.3 | 10.7 | 4.4 | 1.7 | 2.8 |
| Occasionally | No. | 164 | 466 | 630 | 162 | 187 | 349 |
| | Per cent | 45.2 | 45.0 | 45.0 | 44.6 | 36.1 | 39.6 |
| Abstinent | No. | 90 | 424 | 514 | 164 | 284 | 448 |
| | Per cent | 24.8 | 40.9 | 36.7 | 45.2 | 54.8 | 50.9 |
| No information | No. | 25 | 81 | 106 | 21 | 38 | 59 |
| | Per cent | 6.9 | 7.8 | 7.6 | 5.8 | 7.4 | 6.7 |
| Total | No. | 363 | 1036 | 1399 | 363 | 518 | 881 |
| | Per cent | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 |

80 and 57% of the self-poisoning males and females, respectively, were smokers, while this rate was 46 and 41% in the control males and females (Table X).

**Table X**

*Per cent of smokers among self-poisoning and matched control persons at the time of the epidemiological study*

| Group | | Self-poisoning | | | Matched control | | |
|---|---|---|---|---|---|---|---|
| | | Male | Female | Together | Male | Female | Together |
| Smoker | No. | 291 | 585 | 876 | 166 | 212 | 378 |
| | Per cent | 80.2 | 56.5 | 62.6 | 45.7 | 40.9 | 42.9 |
| Total | No. | 363 | 1036 | 1399 | 363 | 518 | 881 |

The medical interventions studied did not show a significant increase in the study sample as compared with the data of the control group (Table XI).

These data e.g. the somewhat lower socio-economic status and the higher rate of alcohol consumption and smoking of self-poisoning persons have confirmed the previous observations [20, 25]. Obviously, some of the above-mentioned variables may have an effect on the pregnancy outcome. Still, owing to the negative results of our study, a separate evaluation of these confounding factors did not seem necessary.

**Table XI**

*Medical interventions in the study and control samples*

| Medical intervention | Self-poisoning | | | | | |
|---|---|---|---|---|---|---|
| | Male | | Female | | Together | |
| | No. | per cent | No. | per cent | No. | per cent |
| X-ray or isotope therapy | 0 | 0 | 5 | 0.5 | 5 | 0.4 |
| Chronic drug treatment under physicians guidance | 45 | 12.4 | 132 | 12.7 | 177 | 12.7 |
| Previous hospitalization except for the event studied | 82 | 22.6 | 233 | 22.5 | 315 | 22.5 |
| Total | 363 | 100.0 | 1036 | 100.0 | 1399 | 100.0 |

| Medical intervention | Matched control | | | | | |
|---|---|---|---|---|---|---|
| | Male | | Female | | Together | |
| | No. | per cent | No. | per cent | No. | per cent |
| X-ray or isotope therapy | 8 | 2.2 | 11 | 3.5 | 19 | 2.2 |
| Chronic drug treatment under physicians guidance | 39 | 10.7 | 66 | 20.8 | 105 | 11.9 |
| Previous hospitalization except for the event studied | 74 | 20.4 | 124 | 39.0 | 198 | 22.5 |
| Total | 363 | 100.0 | 318 | 100.0 | 881 | 100.0 |

*Pros and cons of the human self-poisoning model*

The advantages of a study of the possible mutagenic effect of chemicals in self-poisoning persons are as follows.

1. A great number of cases of attempted suicide by chemicals is available in the toxicological departments.

2. There is an opportunity for a planned, well-organized and continuous study of suicide attempters, as opposed to the unexpected and mostly single occurrence of accidents.

3. The patients in hospital are thoroughly examined thus for example series of blood samples are available and there is no need for special organisation.

4. In general, the quantitative estimates of exposure can be solved. Partly the quantitative values of chemicals in blood, partly in general the severity of clinical symptoms, partly the case history of surviving patients can offer a good basis for estimation of the dose as well as of the type of compounds.

5. As suicides are attempted by different doses of chemicals there is a possibility to study the correlation between dose and response.

Of course, this approach as all the others has some limitations and drawbacks as well.

a) The persons or study samples of those who have attempted suicide cannot be considered to be representative for the whole population.

b) The use of extremely large doses is not frequent and an extrapolation from the large doses to the more common low ones may sometimes raise problems. The generalized dose-response curve has five main regions (Fig. 4).

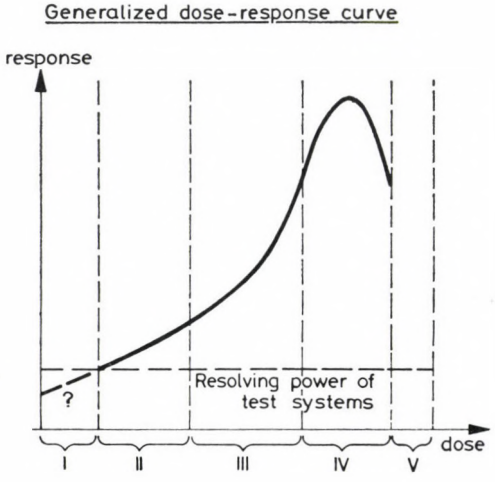



*Fig. 4*

The Ist region at very low doses is mostly inaccessible to practical experiments. The region II at low-intermediate doses has a predominating linear correlation. The region III with intermediate-high doses shows a more or less pronounced exponential rise. The region IV at very high doses shows a fall of the response. The region V with lethal doses elicits no response any more. In general, the cases of attempted suicide are to be found in the regions III to V.

c) Treatment of these patients with antidotes and other drugs may cause some confounding effects.

d) Chemicals are often combined in self-poisoning thus it is difficult to study a single compound. On the other hand it provides a chance to study the interaction of chemicals.

e) The persons who attempt suicide often use continuous medication.

f) These persons, in general consume alcoholic beverages and are smokers.

g) Repeated self-poisonings are not infrequent and may confuse the consequences of different events.

Evaluating the pros and cons, this human "experimental" epidemiological model seems to be practical.

It is a general experience that there is a considerable social hostility against suicide attempters (Robertson, 1977). They do not inspire public concern, they are depressing and unrewarding to deal with. This may be one of the explanations that no study of the possible mutagenic damage has been done in self-poisoning persons and their offspring.

## REFERENCES

1. Bao, T. van, Szabó, I., Ruzicska, P., Czeizel, A.: Chromosome aberrations in patients suffering acute organic phosphate insecticide intoxication. Humangenetik **24** 33–57 (1974)
2. Bridges, B. A., Bochkov, N. P., Jansen, J. D.: Genetic monitoring of human populations accidentally exposed to a suspected mutagenic chemical. Mut. Res. **64** 57—60 (1979)
3. Czeizel, A.: Complex epidemiological approach to human mutagenesis. In: Alacevic, M. (ed.): Progress in Environmental Mutagenesis. Elsevier /North Holland, Amsterdam Biomedical Press 1980, pp. 297–311.
4. Czeizel, A.: Cytogenetic studies as practical procedures for identifying and evaluating genetic risk. In: Bora, K. C. (ed): Chemical Mutagenesis, Human Population Monitoring, and Genetic Risk Assessment. Elsevier/North Holland, Amsterdam 1980.
5. Czeizel, A.: Genetic effect of self-poisoning. In: Sugimura, T., Kondo, S., Takebe, M. (eds): Environmental Mutagens and Carcinogens. Alan R. Liss Inc. New York 1982, pp. 638–656.
6. Czeizel, A., Szentesi, I., Molnár, G.: Effect of self-poisoning on subsequent reproductive outcome. Mut. Res, 1984 (In press)
7. Evans, H. J., O'Riordan, M. L.: Human peripheral blood lymphocytes for the analysis of chromosome aberrations in mutagen tests. Mut. Res. **31,** 135–148 (1975).
8. Graham, J. D. P., Hitchens, R. A. N., Marshall, R. W., Mitchell, G. M.: Self-poisoning — a decennial survey from Cardiff. Publ. Hlth. Lond. **93,** 223–229 (1979).
9. ICPEMC, Report of Epidemiological Committee Mut. Res. **123,** 1—11 (1983).
10. Kessel, N.: Self-poisoning. Brit. med. J. **2,** 1265–1270; 1336—1340 (1965).
11. Molnár, G., Szentesi, I., Czeizel, A.: Chromosome studies in self-poisoning persons. (In Hungarian) Orv. Hetil. **110,** 2941–2942 (1978).

12. Mulvihill, J. J.: Towards documenting human germinal mutagens: epidemiologic aspects of ecogenetics in human mutagenesis. In: Sugimura, T., Kondo, S., Takebe, H. (eds): Environmental Mutagens and Carcinogens. Alan. R. Liss, New York 1982, pp. 625–637.
13. O'Brien, J. P.: Increase in suicide attempts by drug ingestion. The Boston experience, 1964–1972. Arch. gen. Psychiat. **34,** 1165–1169 (1977).
14. Parkin, D., Stengel, E.: Incidence of suicidal attempts in an urban community. Brit. med. J. **2,** 133–138 (1965).
15. Patel, N. S.: A study on suicide. Med. Sci. Law **14,** 129–136 (1974).
16. Patel, A. R.: Attitudes towards self-poisoning. Brit. med. J. **1,** 426–429 (1975).
17. Perry, P., Wolff, S.: New Giemsa method for the differential staining of sister chromatids. Nature (Lond.) **251,** 156–158 (1974).
18. Raposa, T.: Sister chromatid exchange studies for monitoring DNA damage and repair capacity after cytostatics in vitro and in lymphocytes of leukaemic patients under cytostatic therapy. Mut. Res. **57,** 241–251 (1978).
19. Robertson, J.: The epidemiology of self-poisoning, Publ. Hlth. (Lond.) **91,** 75–82 (1977).
20. Schneidman, E. S., Farberow, N. L.: Clues to Suicide. McGraw-Hill New York 1961.
21. Schneidman, E. S. (ed.): Suicidology. Contemporary Developments. Grune and Stratton, New York 1976.
22. Smith, A. J.: Self-poisoning with drugs: a worsening situation. Brit. med. J. **2,** 157–159 (1972).
23. Szentesi, I., Molnár, G., Thész, S., Ruzicska, M., Pál, B. and Czeizel, A.: Chromosome study on patients attempting suicide with drugs (Cytogenetic model of "experimental" mutagenesis in man.) XIVth Int. Congr. Genetics, Moscow, Abstracts, 1978 Part 1, p. 345.
24. Vogel, F., Jager, P.: The genetic load of a human population due to cytostatic agents. Hum. Genet. **7,** 287–304 (1969)
25. Wekstein, L.: Handbook of Suicidology. Principles, Problems and Practice. Brunner/ Mazel, New York 1980.

*Acta Medica Hungarica, 41 (2—3), pp. 93—102 (1984)*

# *Haematology*

## HAEMOSTASIS IN DIABETICS

Maria Misz, Piroska Beck, M. Udvardy, K. Rák

SECOND DEPARTMENT OF MEDICINE, UNIVERSITY MEDICAL SCHOOL, DEBRECEN, HUNGARY

Received: July 10, 1982

Haemostasis was studied in 34 diabetic patients with and without detectable vascular complications (micro- and macroangiopathy). Information on platelet functions was obtained by beta-thromboglobulin determination, and of heparin-thrombin coagulation time, platelet aggregation in vivo, and on the condition of the vessel walls by estimation of factor VIII-protein (VIIIR:Ag). The results were suggestive of an increased platelet activity, the most marked abnormalities having been found in cases of angiopathy. Attention is drawn to the therapeutic possibilities offered by studies of the pathogenetic role of the abnormalities of haemostasis in diabetes.

**Keywords:** diabetes mellitus, micro- and macroangiopathy, thrombocyte function, VIII related antigen (VIIIR:Ag)

### Introduction

Arterial disease in the form of acute arterial occlusion, or of chronic angiopathy (disease of small and large arteries), is common in diabetics. Haemostasis, in the first place the platelet function, in diabetics, has been the subject of extensive studies. Changes in various platelet functions including an increase in platelet-reactivity, have been found by several authors, and abnormalities of the clotting and fibrinolytic systems resulting in enhanced haemostasis have also been described.

There is mounting evidence in support of an involvement of the haemostatic systems, particularly of platelet hyperfunction, in the pathogenesis of atherosclerosis [23, 40]. In view of the close association of diabetes with atherosclerosis, i.e. with macroangiopathy, it is not surprising that the possibility of an altered platelet function in the aetiology of microangiopathy, the other typical corollary of diabetes, has also been raised [9, 11, 33, 41]. It is still uncertain whether hyperreactivity is an intrinsic property of the platelets, or it is conferred on them by some plasma factor. The possibility that the abnormal platelet function is a consequence rather than a causal factor of microangiopathy has also been suggested, a view which has found

Send offprint requests to Dr. Mária Misz, Second Department of Medicine, University Medical School, H-4012 Debrecen, Nagyerdei krt 98, Hungary

large support [1, 39]. But even if this claim is valid, the pathogenetic role of platelet functions, not in the production, but rather in the maintenance and progression of angiopathy, deserves close attention.

The following parameters of haemostasis have been extensively studied in diabetics and found abnormal by several authors.

Platelet adhesiveness measured in vitro (generally by adherence to glass beads) has been found excessive by the majority of workers [21, 22, 32, 43], but this method is not reliable, its result is affected by aggregation, in addition to adhesion. Hyperadhesiveness has been connected with increased plasma levels of the Willebrand-factor in diabetes [8, 9, 30, 37], this humoral factor being closely involved in platelet adhesion.

The results of the aggregation test are also consistently positive. Hypersensitivity of the platelets to ADP was demonstrated many years ago [4], particularly in cases of advanced retinopathy [21], but also in the absence of vascular disease [9, 10, 41]. The view that the primary cause lies not in the platelets but rather in a platelet-stimulating plasma factor gaining access to the circulation, has been voiced by several authors [11, 12, 29]. This plasma factor has been identified lately with immunocomplexes [13] stimulating the aggregation and release of platelets. The immunocomplexes may play a part in the pathogenesis of diabetic angiopathy, taking effect by the activation of the platelets [14].

Secondary platelet hyperaggregation is inhibited by aspirin, an inhibitor of prostaglandin-synthetase (cyclo-oxygenase) [41]: the platelets of diabetics are hypersensitive to arachidonic acid and form prostaglandin-E-like material in increased amounts under the effect of ADP, adrenaline, arachidonic acid and thrombin [18]. The synthesis of thromboxan-$A_2$ ($TXA_2$) is also significantly increased, the increase being related to the blood glucose levels [19]. Impairment of prostacyclin ($PGI_2$) synthesis by the vascular endothelium may also contribute to the platelet abnormalities [20, 44].

Alterations of platelet turnover [10] and elevated plasma levels of the platelet-specific protein, beta-thromboglobulin (BTG), have been observed [5, 39]. This may be connected with the aggregation and release reaction in vivo, in the same manner as the augmented plasma level of platelet factor 4 ($PF_4$) with known antiheparin activity [7].

The protein of factor VIII complex, VIIIR:Ag or Willebrand-factor, has been extensively studied in diabetes, with consistent findings of elevated plasma levels. The first important observation was that the abnormal haemostasis in Willebrand's disease responds better to the plasma of diabetics than of nondiabetic patients [17]. This factor attains the highest levels in diabetic retinopathy [8], but it is increased in chemical diabetes as well [9, 30] and declines in response to strict dietary and drug control of glucose metabolism [7, 38]. VIIIR:Ag originates for the greatest part from the endothelium, only

15% of the circulating antigen comes from the megakaryocyte-platelet system [28]. This factor plays an essential part in haemostasis under both physiological and pathological conditions. It was, therefore, justified to look for causal relationships between the raised levels and angiopathy. Though we lack any conclusive evidence in this respect, a high concentration of the Willebrand-factor is none the less regarded as a sign of permanent damage to the vascular endothelium [38].

The relationships between the abnormalities of haemostasis, first of all those of platelet functions and vascular complications in diabetes, have yet to be clarified, and we are still unable to assess the possible benefits of the present therapeutic attempts directed at these abnormal functions.

The aim of the present study was to throw light on the relationship between haemostasis and angiopathy in diabetics.

## Materials and methods

Thirty-four diabetic patients aged from 26 to 60 years were studied. Apart from insulin or oral antidiabetic agents drugs were avoided.

Group I included 16 patients known to have diabetes for 2 to 5 years; 5 of them were on insulin, 11 on oral antidiabetics, in addition to the diet. The history, clinical features, ECG, ophthalmoscopy, oscillometry and renal studies ruled out the presence of considerable vascular disease in all of the cases. In the 18 patients of Group II the disease had been known for more than 5 years; 8 patients were on insulin, the other 10 on oral antidiabetics, in addition to the diet. The investigations revealed vascular complications (macro- and microangiopathy).

*Beta-thromboglobulin* (BTG) was determined from platelet-poor plasma by radioimmunoassay (RIA kit, Radiochemical Centre, Amersham). The mean plasma BTG level of 30 non-diabetic subjects with no apparent vascular disease was $21 \pm 4.1$ ng/ml.

The *heparin-thrombin coagulation time* (HTCT) measures platelet factor 4 indirectly. A radioimmunoassay for the measurement of the heparin neutralizing factor of platelets (Abbot) was outside of our facilities, so the indirect assay was performed by the method of O'Brien with some modifications [34]. A thrombin solution causing the control plasma to coagulate in 20 s is prepared, and heparin is added to the plasma in an amount to give a coagulation time of $40 \pm 1.5$ s. The higher the antiheparin activity of the unknown plasma, the shorter the coagulation time with the same thrombin solution.

For quantitative determination of *circulating platelet-aggregates* the method of Wu and Hoak [45] modified by Schmidt [42] was used. The method permits to define the platelet-aggregate ratio. Venous blood is drawn with minimum stasis, one part directly into an EDTA-formalin solution, the other into EDTA, and kept at room temperature for 15 min. The aggregates are fixed by formalin, but they disaggregate in EDTA. After low-speed centrifugation to sediment, the erythrocytes, leukocytes and fixed aggregates, the platelet count was determined from the uppermost layer of the sample by direct phase-contrast microscopy. The platelet-aggregate ratio is the quotient of the platelet count in EDTA-formalin and platelet count in EDTA. The ratio in 20 controls was $0.99 \pm 0.07$. If aggregates are formed in the circulating blood, the platelet count in the formalin sample declines as compared with the EDTA-sample and the values of the platelet-ratio decrease.

*VIIIR: Ag* was determined by the rocket technique of Laurell, on the basis of quantitative immunoelectrophoresis [46]. The normal value was $113 \pm 40\%$. The clotting activity of factor VIII (*VIII:C*) was determined on the basis of a monophasic test by means of plasma deficient in factor-VIII (Goedecke) and of activated partial thromboplastin (Platelin, Goedecke), using a semi-automatic coagulometer (Clotek, Hyland). The mean value in 10 controls was $84 \pm 3$. The VIIIR:Ag/VIIIC-ratio, calculated in 10 controls, was $1.34 \pm 0.48$.

## Results

Elevated plasma BTG levels were found in both groups, i.e. in the patients with no vascular complications (NVC) and in those with vascular complications (VC). The difference between the two groups (70.3 ± 19.2 vs. 156 ± 52.5 ng/ml) was highly significant (Fig. 1).

The heparin-thrombin coagulation time in Group I (39 ± 3.3) failed to differ significantly from the control group. Shortened coagulation times were

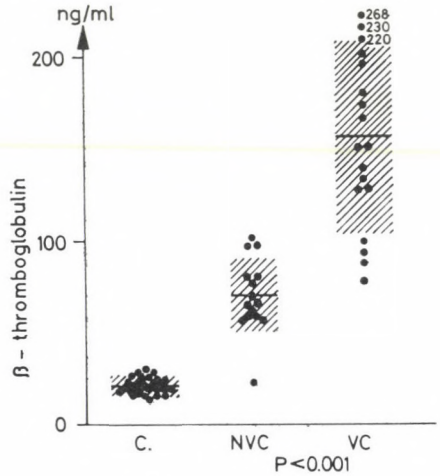



*Fig. 1.* Beta-thromboglobulin levels in diabetics with no vascular complications (NVC), with vascular complications (VC) and in the controls (C)
mean ± S. D. (hatched area)

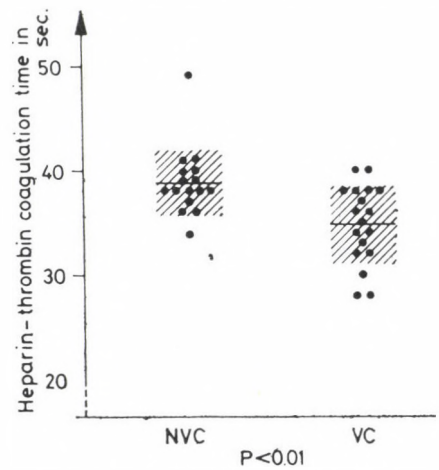



*Fig. 2.* Heparin-thrombin coagulation time in diabetics with no vascular complications (NVC) and with vascular complications (VC)
mean ± S. D. (hatched area)

found in Group II (35 ± 3.7 s). The difference between the two groups was slightly significant (Fig. 2).

The VIIIR:Ag levels were augmented in both groups, the mean values being 133 ± 47 for Group I and 248 ± 125 for Group II. The differences between the two groups and between Group II and the controls were significant. The VIII:C values gave no evaluable differences between the control group and the two study groups. The mean VIIIR:Ag/VIII:C ratio was 1.52 ± 7 in the 10 NVC patients of Group I and 2.79 ± 1.08 in the 14 VC patients of Group II; the difference was significant (Fig. 3).

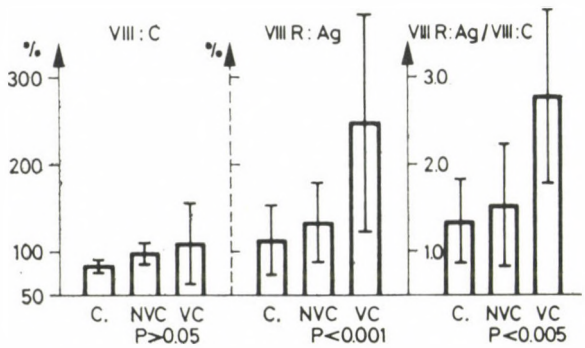



*Fig. 3.* VIII:C, VIIIR : Ag levels and the quotient of the two values in diabetics with no vascular complications (NVC), with vascular complications (VC) and in the controls (C) mean ± S. D.

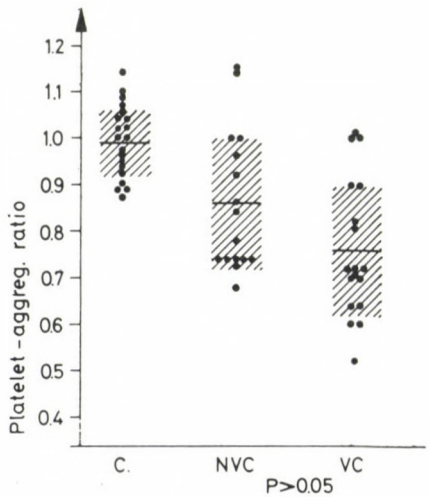



*Fig. 4.* Platelet-aggregation ratio in diabetics with no vascular complications (NVC), with vascular complications (VC) and in the controls (C) mean ± S. D. (hatched area)

The platelet-aggregation ratio (aggregation-index) was lower in the diabetic groups than in the controls where it approximated 1.0. The reduction was more marked in the VC group. The difference between Group II and the controls was significant, between Groups I and II it was not significant (Fig. 4).

## Discussion

Though the last decades have witnessed considerable advances in the therapy of diabetics, their life expectation, particularly in the insulin-dependent form, is far below that of the general population. This is due in the first place to the associated macro- and microangiopathy. Renal failure and acute cardiac death also contribute to the mortality and blindness is a common complication.

The role of platelets in the pathogenesis of diabetic vascular disease has been receiving increasing attention. A platelet hyperfunction has been found by several workers, and the biochemical mechanism underlying these abnormalities is also under investigation. It is sought to establish the value of anti-platelet drugs, even though the relationships between abnormal platelet functions and diabetic vascular disease are still far from being clarified. Nor is it certain whether in vitro assays reflect abnormalities in vivo. The increased platelet turnover [10, 15] as well as the elevated plasma concentration of certain platelet-specific proteins, in particular of BTG, do, however, point to relationships of this kind. But even in this case, neither the abnormal platelet function, nor the increase in prostaglandin synthesis should be considered characteristic of the diabetes itself. Both may be secondary to the vascular disease and to the shortened platelet life-span. In diabetes the percentage of young platelets, often of macrothrombocytes, is increased [15]. These cells are hypersensitive to ADP and adrenaline, they form more prostaglandin-endoperoxide and more malonyldialdehyde than normally, and are characterized by a metabolic hyperactivity [26]. The question whether the increased platelet function was the cause or rather a consequence of angiopathy, or even whether there was any causal connection between the two, has remained largely unanswered.

It is generally accepted that an increased plasma BTG level reflects in vivo platelet aggregation and release. Abnormally high levels in diabetes have been found by the majority of workers, but there is also evidence to the contrary. Campbell et al. [6] observed normal values even in diabetes with VC and alleged for the explanation of their findings that if aggregates are confined to a small proportion of the vasculature, the platelets involved in the process are small in number, therefore too little BTG is released to be demonstrable by the method used. Moreover, the turnover of platelets taking part

in the aggregates is small, and when the stores are depleted, the plasma-BTG remains low, owing to the comparatively short half-life of platelets. While the test fails to indicate a minor local platelet aggregation, it shows high levels in the case of generalized platelet activation, that is, under conditions of hypercoagulability.

In the present study increased plasma concentration have been found in the absence as well as in the presence of vascular disease (Groups I and II), but the levels were significantly higher in Group II than in Group I. According to data in the literature the BTG level is not related to the blood glucose level or to the proportion of glycosylated haemoglobin ($HbA_{1C}$), but depends on the lipid levels and the condition of the vessels. The highest levels have been found in cases associated with renal failure or with retinopathy [3, 31]. The results of BTG determination give the strongest support to the claim that an abnormal platelet function contributes to the development of vascular disease in diabetes.

The $PF_4$-levels are also increased [3, 7]. This factor has been found similar in behaviour to BTG, but the pertaining data are sparse. In the present study the reduction in heparin-thrombin coagulation time was confined to the group with vascular complications. Attention has been drawn recently to the informative value of parallel measurements of BTG and $PF_4$. Increased levels of the two proteins are indicative of platelet activation in vivo. The half-life of $PF_4$ being shorter, the concentration of BTG is higher, so the BTG/$PF_4$ ratio is also high [25]. If only one of the factors or activities increases, an artifact may be suspected in all probability due to the release of the factor in vivo.

The protein of factor VIII complex (VIIIR:Ag) has been widely studied. Its level is abnormally high in diabetics. This is independent from the metabolism of glucose but it increases with the severity of vascular disease. The highest concentrations were found in diabetic retinopathy [2, 38]. It is unrelated to $HbA_{1C}$, but the level may decline in response to dietary or antidiabetic treatment [8]. We found increased plasma concentrations in both groups, but the values in Group II were significantly higher than those in Group I. A mean value of 219% was found in 113 unselected adult diabetics. We have noted similarly high values (221%) in 28 cases of acute myocardial infarction, a mean value of 193% in 30 cases of atherosclerosis (unpublished data). The plasma VIIIR:Ag-level indicates a permanent vascular damage. Its increase is a secondary manifestation. The factor may, however, play a part in the maintenance and progression of the angiopathy.

An abnormally low level of factor VIII:RAg, thus of Willebrand's factor or of the ristocetin cofactor (Willebrand's disease) results in an impaired platelet adhesion. Abnormally high levels are, on the other hand, obviously associated with hyperadhesiveness. Assays for this factor are very cumber-

some. An increase in bleeding time has been reported in diabetes [24], but even this apparently simple issue has yet to be ascertained. The increased VIIIR:Ag-levels do not seem to be responsible for the hyperaggregative tendency demonstrable already in the early stage [38].

An increase in the VIIIR:Ag/VIII:C quotient has been found by several authors, particularly in the case of complications [2, 7, 16]. It is assumed that the vascular endothelium produces VIIIR:Ag only and VIII:C joins the molecule at a later stage. It should be remembered that even small amounts of intravasal thrombin are highly destructive to VIII:C. We lack precise knowledge about the stimulus eliciting the release of the antigen of factor VIII from the endothelium. According to several observations, the plasma level of the Willebrand-factor correlates with the growth hormone level in diabetes [2, 36].

We have not studied the aggregation tendency of platelets in vitro, seeking instead to quantify the aggregates formed in vivo. In the VC patients the ratio was significantly lower than in the controls. This finding also points to an activation of the platelet system.

The present results have furnished evidence relating to the connection between haemostasis, in particular platelet functions, and the vascular disease of diabetics. Though all the observed abnormalities may be secondary to the vascular process, yet the increased BTG-level in diabetes even in the absence of any apparent angiopathy, points to a primary alteration of the platelet functions. Therapeutic attempts directed against this hyperfunction are justified, even if the role of the abnormal platelet activity is confined to the maintenance and progress of the angiopathy [27].

From the present findings the following conclusions may be drawn:

1. The high beta-thromboglobulin level, the reduced heparin-thrombin coagulation time (thus an increased plasma activity of platelet factor 4), and the reduced platelet-aggregation ratio (i.e. the enhanced aggregation tendency of platelets in vivo), found in early, particularly in juvenile, diabetes, reflect a hyperfunction of the platelet system and suggest a possible role of abnormal platelet functions in the production of angiopathy. For this reason drug therapy directed at platelet hyperfunction seems promising and is, therefore, justified.

2. High VIIIR:Ag-concentrations are suggestive of extensive angiopathy. Determination of this factor is of diagnostic value. High levels imply the necessity for dietary restrictions and/or drug therapy.

3. Our results, though obtained in a small number of cases, highlight the need for further studies of haemostasis in diabetics, in order to gain closer insight into the pathogenesis of atherosclerosis.

## REFERENCES


1. Arkin, C. F., Hartman, A. S.: The hypercoagulability states. Crit. Rev. clin. Lab. Sci., **10,** 379–428 (1979).
2. Bensoussan, D., Levy-Taledano, S., Passa, F., Caen, J., Canivet, J.: Platelet hyperaggregation and increased plasma level of von Willebrand factor in diabetics with retinopathy. Diabetologia **11,** 307–312 (1975.)
3. Betteridge, D. J., Zahavi, J., Jones, N. A. G., Shine, B., Kakkar, V. V., Galton, D. J.: Platelet function in diabetes mellitus in relationship to complications, glycosylated haemoglobin and serum lipoproteins. Eur. J. clin. Invest. **11,** 273–277 (1981).
4. Breddin, K.: Experimental and clinical investigations on the adhesion and aggregation of human platelets. Exp. Biol. Med. **3,** 14—23 (1968).
5. Burrows, A. W., Chavin, S. I., Hockaday, T. D. R.: Plasma-beta-thromboglobulin concentrations in diabetes mellitus. Lancet **1,** 235–237 (1978).
6. Campbell, I. W., Dawes, J., Fraser, D. M., Pepper, D. S., Clarke, B. F., Duncan, L. J. P., Cash, J. D.: Plasma beta-thromboglobulin in diabetes mellitus. Diabetes **26,** 1175–1177 (1977).
7. Chmielewsky, J., Fabriszewsky, R.: Platelet factor IV ($PF_4$) release during human platelet aggregation in diabetic patients. Thromb. Diath. haemorrh. (Stuttg.) **24,** 203–205 (1970).
8. Coller, B. S., Frank, R. N., Milton, R. C., Grelnick, H. R.: Plasma cofactors of platelet function: correlation with diabetic retinopathy and haemoglobins $A_{1C}$. Ann. intern. Med., **88,** 311–316 (1978).
9. Colwell, J. A., Halushka, P. V., Sarji, K., Levine, J., Sagel, J., Nair, R. M. G.: Altered platelet function in diabetes mellitus. Diabetes **25,** Suppl. 2, 826–831 (1976).
10. Colwell, J. A., Sagel, J., Crook, L., Chambers, A., Laimins, J.: Correlation of platelet aggregation, plasma factor activity, and megathrombocytes in diabetic subjects with and without diabetic vascular disease. Metabolism **26,** 279–285 (1977).
11. Colwell, J. A., Halushka, P. V., Sarji, D. E., Lopesvirella, M. F., Sagel, J.: Vascular disease in diabetes: pathophysiological mechanisms and therapy. Arch. intern. Med. **139,** 225–230 (1979).
12. Colwell, J. A., Nair, R. M. G., Halushka, P. V., Rogers, C., Whetsell, A., Sagel, J.: Platelet adhesion and aggregation in diabetes mellitus. Metabolism **28,** Suppl. 1, 394–400 (1979).
13. Colwell, J. A., Van Zile, J., Kilpatrick, J. M., Nair, R. M. G., Virella, G.: Plasma factors and platelet aggregation in diabetes mellitus. In: Pathogenetic Concepts of Diabetic Microangiopathy. Ed.: Standl, E., Mehnert, H., Horm. Metab. Res. Suppl. Ser. **11,** 1–6 (1981).
14. DiMario, U., Iavicoli, M., Andreani, D.: Circulating immune complexes in diabetes. Diabetologia **19,** 89–92 (1980).
15. Garg, S. K., Lackner, H., Karpatkin, S.: The increased percentage of megathrombocytes in various clinical disorders. Ann. intern. Med. **77,** 361–369 (1972).
16. Gensini, G. F., Abbate, R., Favilla, S., Neri Serneri, G. G.: Changes in platelet function and blood clotting in diabetes mellitus. Thromb. Haemostas. **42,** 983–993 (1979).
17. Gonzalez, J., Colwell, J. A., Sarji, K. E., Nair, R. M. G., Sagel, J.: Effect of metabolic control with insulin on plasma von Willebrand factor activity (VIIIR:WF) in diabetes mellitus. Thromb. Res. **17,** 261–266 (1980).
18. Halushka, P. V., Lurie, D., Colwell, J. A.: Increased synthesis of prostaglandin-E-like material by platelets from patients with diabetes mellitus. New Engl. J. Med. **297,** 1306–1310 (1977).
19. Halushka, P. V., Colwell, J. A., Rogers, C.: Increased thromboxane ($TXB_2$) synthesis by platelets from patients with diabetes mellitus (DM): studies with a TX synthetase inhibitor and a TX receptor antagonist. Clin. Res. **27,** 296A (1979).
20. Harrison, H. E., Reece, A. H., Johnson, M.: Decreased vascular prostacyclin in experimental diabetes. Life Sci. **23,** 351–355 (1978).
21. Heath, H., Bridgen, W. D., Canever, J. V., Pollock, J., Hunter, P. R., Kelsey, J., Bloom, A.:Platelet adhesiveness and aggregation in relation to diabetic retinopathy. Diabetologia **7,** 308–315 (1971)
22. Hellem, A. J.: Adenosine diphosphate induced platelet adhesiveness in diabetes mellitus with complications. Acta med. scand. **190,** 291—295 (1971).
23. Hess, H.: Pro-Contra: Entstehung der Arteriosklerose. Internist **22,** 658–663 (1981).
24. Joist, J. H., Baker, R. K., Smith, M.: Cit. Paton, R. C. et al. [38]

25. Kaplan, K. L., Owen, J.: Plasma levels of beta-thromboglobulin and platelet factor 4 as indices of platelet activation in vivo. Blood **57,** 199–202 (1981).
26. Karpatkin, S.: Heterogeneity of human platelets. I. Metabolic and kinetic evidence suggestive of young and old platelets. J. clin. Invest. **48,** 1073–1082 (1969).
27. Klaff, L. J., Kernoff, L., Vinik, A. I., Jackson, W. P. U.: Sulfonylureas and platelet function. Amer. J. Med. **70,** 627–630 (1981) .
28. Koutts, J., Howard, M. A., Firkin, B. G.: Factor VIII physiology and pathology in man. Progr. Hemat. **11,** 115–144 (1979).
29. Kwaan, H. C., Colwell, J. A., Cruz, S., Suwanwela ,N., Dobble, J.: Increased platelet aggregation in diabetes mellitus. J. Lab. clin. Med. **80,** 236–246 (1972)
30. Lufkin, E. G., Fass, D. N., O'Fallon, W. M. Bowie, E. J. W.: Increased von Willebrand factor in diabetes mellitus. Metabolism **28,** 63–66 (1979).
31. Matthews, J. H., O'Connor, Hearnshow, J. R., Wood, J. K.: Beta-thromboglobulin and glycosilated haemoglobin in diabetes mellitus. Scand. J. Haemat. **23,** 421—426 (1979)
32. Mayene, E. E., Bridges, J. M., Weaver, J. A.: Platelet adhesiveness, plasma fibrinogen, and factor VIII levels in diabetes mellitus. Diabetologia **6,** 636–640 (1970).
33. McMillan, D. E.: Deterioration of the microcirculation in diabetes. Diabetes **24,** 944–957 (1975).
34. Misz, M., Boda, Z., Rák, K.: Study of heparin neutralizing effect of plasma in haemostatic disorders (in Hungarian). Orv. Hetil. **118,** 2195–2199 (1977).
35. Odegaard, A. E., Skalhegg, D. A., Hellem, A. J.: Increased activity of "anti-Willebrand factor" in diabetic plasma. Thrombos. Diathes. haemorrh. (Stuttg.) **11,** 27–37 (1964).
36. O'Malley, B. C., Timperley, W. R., Ward, J. D., Porter, N. R., Preston, F. E.: Platelet abnormalities in diabetic peripheral neuropathy. Lancet **2,** 1274–1276 (1975).
37. Pandolfi, M., Almér, L. O. Holmberg, L.: Increased von Willebrand antihaemophilic factor A in diabetic retinopathy. Acta ophthal. (Kbm.) **52,** 823–829 (1974).
38. Paton, R. C., Passa, Ph., Canivet, J.: Von Willebrand factor, diabetes mellitus and retinopathy. In: Pathogenetic concepts of diabetic microangiopathy. Horm. Metab. Res. Suppl. Ser. **11,** 55–58 (1981).
39. Preston, F. E., Ward, J. D., Marcola, B. H., Porter, N. R., Timperley, W. R., D Malley, B. C.: Elevated beta-thromboglobulin levels and circulating platelet aggregates in diabetic microangiopathy. Lancet **1,** 238–239 (1978).
40. Ross, R., Glomset, J. A.: The pathogenesis of atherosclerosis. New Engl. J. Med. **295,** 420–425 (1975).
41. Sagel, J., Colwell, J. A., Crook, L., Laimins, M.: Increased platelet aggregation in early diabetes mellitus. Ann. intern. Med. 82, 733–738 (1975).
42. Schmidt, K. G.: Diagnostic usefulness of a newly described method for the quantitative determination of platelet aggregates in cases of deep venous thrombosis. Dan. med. Bull. **23,** 249–250 (1976).
43. Shaw, S., Pegrum, G. D., Wolff, S., Ashton, W. L.: Platelet adhesiveness in diabetes mellitus. J. clin. Path. **20,** 845–847 (1967).
44. Silberbauer, K., Schernthaner, G., Sinzinger, H., Piza-Katzer, H., Winter, M.: Decreased vascular prostacyclin in juvenile-onset diabetes. New Engl. J. Med. **300,** 366–367 (1979).
45. Wu, K. K., Hoak, J. G.: A new method for the quantitative detection of platelet aggregates in patients with arterial insufficiency. Lancet **2,** 924–926 (1974).
46. Zimmerman, T. S., Hover, L. W., Dickson, L., Edgington, T. S.: Determination of the von Willebrand's disease antigen (factor VIII-related antigen) in plasma by quantitative immunoelectrophoresis. J. Lab. clin. Med. **86,** 152–159 (1975).

*Acta Medica Hungarica, 41 (2—3), pp. 103—112 (1984)*

*Gyneacology*

# RESPONSE OF THE CERVICAL FACTOR TO COMBINED TREATMENT WITH CLOMIPHENE AND ETHINYL-OESTRADIOL

M. Sas, G. Godó, J. Szöllősi

DEPARTMENT OF OBSTETRICS AND GYNAECOLOGY, UNIVERSITY MEDICAL SCHOOL, SZEGED, HUNGARY

Received: July 7, 1982

Combined clomiphene + ethinyl-oestradiol therapy was applied to 103 women with functional infertility. Ovulatory responses were obtained with clomiphene in a number of cases, but no pregnancy ensued. Ethinyl-oestradiol was then administered in low doses together with clomiphene over 3 cycles until the day before the expected date of ovulation. This resulted in a further increase in the number of ovulatory cycles, and one-third of the women had become pregnant. Comparing the two types of treatment on the basis of quality, quantity and sperm-penetration of the cervical mucus, low doses of oestradiol were found to affect these parameters favourably. If the treatment is unsuccessful, i.e. if the parameters of mucus or of the conditions of penetration fail to respond, further investigations are required to find out whether a permanent damage to the cervical epithelium or some penetration disorder of immunological origin is reponsible for the infertility.

**Keywords:** infertility, cervical factor, clomiphene, ethinyl oestradiol

## Introduction

Hypoplasia, surgical or obstetric injuries, strictures of the uterine cervix are common sources of female infertility [15]. Cyclic changes in the amount and biochemical characters of cervical mucus play a decisive part in impregnation. The mucus of the cervical canal is produced by the epithelial cells which form thousands of crypts in the cervical wall [2]. These are essential to the survival and transport of spermatozoa. In the secretory epithelium there are also ciliated cells which presumably play a part in directing the flow of mucus, in other words, of the migration of macromolecules [16].

The secretory activity of the cervical epithelium is under hormonal control. With approaching ovulation the mucus increases in volume, it becomes clear and fluid. At the time of ovulation sperm penetration of the cervical mucus is at its maximum [42]. The daily amount of mucus varies between 20 and 600 mg [25]; its volume and composition depends on the levels of the

Send offprint requests to Dr. M. Sas, Department of Obstetrics and Gynecology, University Medical School, H-6701 Szeged, Semmelweis u. 1., Hungary

regulatory hormones, on the number and hormone-sensitivity of the secretory units. The cervical mucus is of aqueous character. Its water content is increased by oestrogen, reduced by progesterone. It also contains soluble components such as inorganic salts and carbohydrates, further lipids and proteins including immunoglobulins and enzymes, and mucin macromolecules [4, 8, 10, 34, 38]. It may also contain specific anti-sperm antibodies which either originate from the plasma, or are produced locally, and affect the viability, the migration of spermatozoa.

Involvement of the cervical factor in infertility has been the subject of extensive research [1, 11, 12, 21, 23, 26, 35, 40]. Studies in vitro of sperm penetration of cervical mucus were done first by Miller and Kurzrok [26]. A widely adopted assay based on the follow-up the cervical factor in the successive phases of penetration was described by Jaszczak and Hafez [14].

Inadequacy of the cervical mucus calls for investigation of ovarian function, though it reflects the ovarian cycle less closely than does the condition of the endometrium [10]. It may be e.g. inadequate in normally ovulating females with normal sexual steroid levels.

Observations in connection with clomiphene treatment have shown that the crypts fail to be released from antioestrogen control until shortly before the preovulatory phase [7]. Clomiphene is inhibitory to the cervical crypts and a thick, adhesive mucus interfering with penetration and conception is formed. Though clomiphene represents a definite hallmark in the management of ovulatory disturbances there remained a number of cases in which despite satisfactory ovulatory responses, the rate of pregnancies was lower than expected.

In the present study the effect of oestradiol treatment on the cervical factor and on ovarian function was examined in women who despite a successful ovulatory response to clomiphene, had failed to become pregnant.

## Materials and methods

In women with functional infertility with a normal prolactin level our current practice is to administer clomiphene over three cycles during which information is gained on the pattern of the cycles and tests in vitro for the quality of cervical mucus and for sperm penetration. To 103 women who had failed to conceive under this treatment, ethinyl oestradiol (MIKROFOLLIN mite) was administered in doses of 0.01 to 0.02 mg in addition to clomiphene, and the investigations were continued.

The cycles revealed the following patterns, established by measurement of the basal temperature and by estimation of the progesterone level in the luteal phase.

1. Ovulatory cycles with regular biphasic basal temperature curve and a serum progesterone level over 30 nmol/l;
2. No distinct cycle (basal temperature indistinct, long proliferation phase, short hyperthermic phase, serum progesterone between 15 and 30 nmol/l);
3. An ovulatory cycle (monophasic basal temperature curve, serum progesterone less than 15 nmol/l).

**Table I**

*Parameters and classification of cervical score*

| | 0 | 1 | 2 | 3 |
|---|---|---|---|---|
| Mucus quantity | None | Scanty | Medium: In the external orifice the mucus forms a glistening drop which comes off readily | Plentiful mucus pouring from the external uterine orifice |
| Spinnbarkeit | None | Poor (mucus-thread extensible to ¼ of portio-vaginal distance) | Medium (mucus-thread extensible to ½ of the same distance) | Marked (mucus-thread extensible to the external orifice |
| Arborization | None (amorphous mucus) | None (in places straight lines with no collateral branches) | Partial (pattern of straight lines, in places with collateral branches, in other places amorphous mucus) | Complete (typical fernlike arborization over the entire specimen) |
| Cervix | Closed (pale red mucosa; probe passes the cervical canal with difficulty) | Closed | Partly open (pale red mucosa; cervical canal readily passed by probe) | Patent (hyperaemic mucosa) |

Cervical score and in vitro sperm penetration were studied on the day before the expected date of ovulation, in the early follicular or luteal phase, depending on the aims of the study.

*Study of mucus:*

1. Spinnbarkeit (ductility, its capacity to be drawn out into a thread)
2. Ferning's test (arborization)
3. Viscosity
4. Cell count
5. pH

The *cervical factor* is evaluated by a 12-point score. The volume of mucus, its spinnbarkeit the degree of arborization and the width of the external cervical orifice scored 0 to 3 points each [11, 12]. Classification: 0–3 negative, 4–7 initial, 8–10 good, 11–12 excellent (Table I.).

*In vitro sperm-penetration test:*

1. Slide-test [26]
2. Capillary tube test [19]

Sperm penetration is considered normal if more than 6, unconvincing if 3 to 6, poor if less than 3 motile spermatozoa are found per visual field [24].

During oestradiol treatment the mucus is again scored and the sperm penetration test is repeated in the preovulatory phase. If the results are unsatisfactory, the doses are increased from 0.01 to 0.02 mg. If improvement of the quality of mucus, in the presence of a normal sperm penetration, has been attained by 0.01 mg, the cervical factor is adequate. If the higher dose has to be administered to elicit an adequate response, we deal with a relative dysmucorrhoea.

Earlier inflammatory processes, surgical interventions or other lesions, may affect the number of muciferous units and their responsiveness to hormonal impulses. If high doses of oestradiol also fail to elicit a response, the mucus is impermeable to sperm: absolute dysmucorrhoea. In some cases oestradiol produces mucus of good quality (score over 8), but penetration remains inadequate with husband's and donor's sperm alike. This is a penetration dysmucorrhoea, which may be of immunological origin. If the mucus of the wife is of good quality, penetrable by donor sperm, but the sperm of the husband penetrates the mucus of wife and donor poorly, we have to deal with an inherent sperm-defect, the cause of which may remain unidentified by seminal analysis.

## Results

The results obtained are shown in Table II. The ovulatory response to unsuccessful clomiphene treatment has also been listed. Actually, clomiphene allowed to attain a pregnancy rate of 30% [32]. In infertility due to abnormalities of the cervical factor, thus also in the present material, the pregnancy rate can further be increased by additional oestradiol. A certain increase in the ovulatory rate may be ascribed to a positive feedback mechanism of ethinyl oestradiol on LH-secretion.

**Table II**

*Results obtained with clomiphene and with clomiphene + ethinyl-oestradiol*

| Cycle pattern | Before treatment | Clomiphene | Clomiphene + + oestradiol |
|---|---|---|---|
| Monophasic | 61 | 19 | 11 |
| Indistinct | 23 | 22 | 19 |
| Biphasic | 19 | 62 | 73 |
| | | Pregnancies: 34 (33%) | |

Table III lists the results of sperm penetration tests in vitro. We generally used the capillary tube method, the slide method having been confined to cases in which the mucus was scanty.

**Table III**

*Study of sperm penetration in vitro*

| Grading of penetration | Clomiphene | Clomiphene + + oestrogen |
|---|---|---|
| Good | 31 | 64 |
| Poor | 47 | 27 |
| None | 25 | 12 |

The majority of the patients displayed relative dysmucorrhoea which proved responsive to oestradiol. Failure of the penetration parameters to respond to this treatment calls for differentiation between penetration dysmucorrhoea, absolute dysmucorrhoea and inherent sperm defect, on the basis of crossed tests with donor mucus and sperm, and exclusion of an anatomical malposition.

The diagrams in Figs 1, 2, 3, 4 and 5 represent typical findings and responses to combined therapy, on the basis of case reports.

*Case 1.* The patient aged 26 years had had two improductive marriages. The investigations revealed functional infertility. Clomiphene administered over three cycles was of no benefit. The cycle showed no definite pattern. The preovulatory cervix score and sperm-penetration were inadequate. Administration of ethinyl-oestradiol was followed by a regular biphasic cycle and good penetration (Fig. 1).

*Case 2.* This 28 years old patient had failed to conceive since her last childbirth which had been followed by surgical repair of the portio vaginalis. The cycles in the course of clomiphene

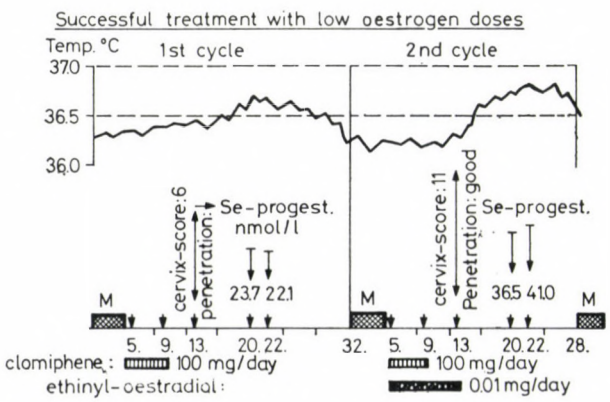



*Fig. 1* **Successful treatment with low dose of ethinyl-oestradiol**

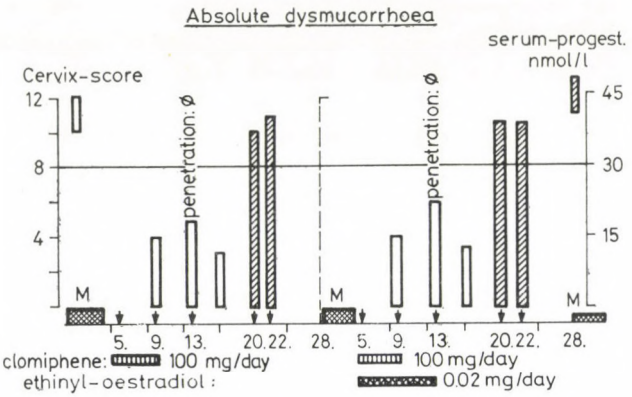



*Fig. 2* Absolute dysmucorrhoea

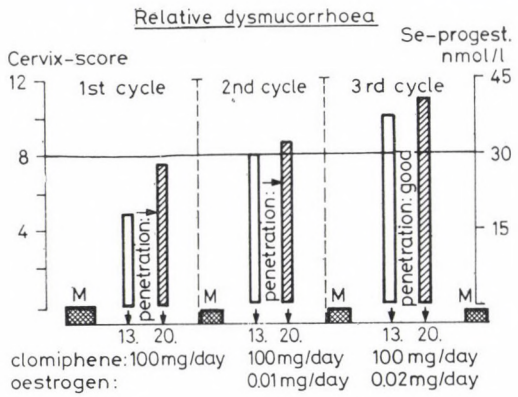



*Fig. 3* Relative dysmucorrhoea

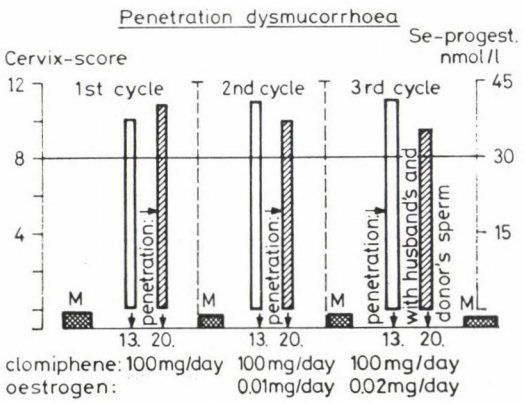



*Fig. 4* Penetration dysmucorrhoea

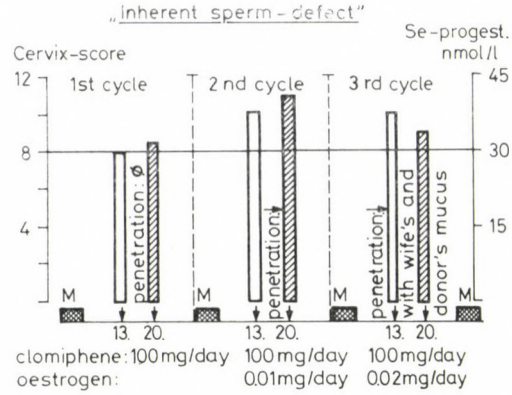



*Fig. 5* Inherent sperm defect

therapy were found to be regular, but the cervical score was low, penetration was absent. Combined clomiphene + ethinyl-oestradiol therapy failed to give any benefit, in all probability because of permanent damage to the muciferous units (Fig. 2).

*Case 3.* A woman aged 22 sought our advice for primary infertility. Her cycles were monophasic. In the course of clomiphene therapy she developed regular ovulatory cycles, but sperm penetration was poor. Clomiphene was combined with ethinyl-oestradiol, the dose of which had to be raised to 0.02 mg to ensure an adequate cervical score and penetration (Fig. 3).

*Case 4.* This patient of 33 years had failed to conceive for years subsequent to two interruptions. Both fallopian tubes were patent. The cervical score was adequate, the husband was fertile, but sperm penetration was inadequate. Combined treatment failed to modify the results of the penetration test to any significant degree, either with husband's or donor's sperm (Fig. 4).

*Case 5.* A couple presented after three years of infertile marriage. With the exception of hypozoospermia of the husband, all findings were negative. Both husband and wife were started on clomiphene, whereupon the husband became normozoospermic. In the course of combined ethinyl-oestradiol therapy sperm penetration of the good quality cervical mucus remained inadequate with the husband's and a donor's sperm. (Fig. 5).

## Discussion

Migration of spermatozoa across the cervical mucus has been extensively studied [20, 28, 39]. Both the slide-test [30] and the capillary tube-test [3, 18, 19] are suitable for the study of sperm-penetration across the mucus which is optimum in the preovulatory phase [42]. The duration of this phase shows wide individual variations. According to Davajan and Nakamura [6] and others, these in vitro tests are best carried out 2 days before the expected rise of body temperature, on the basis of the temperature chart of the previous cycle. The mucus remains penetrable for a period of 4 to 6 days. Inhibition of penetration is thus considerable on the 9th day of the cycle or 1 to 2 days after ovulation [27].

According to Moghissi [27], abnormalities of the cervix or its secretion account for 15% of infertile marriages. Examination of mucus for volume, spinnbarkeit, Ferning's test, cell count and viscosity, provides reliable infor-

mation. The tests are simple and require no expensive equipment. This is also valid for the various penetration tests in vitro.

The cervical score correlates well with oestrogenization, reflected in sperm penetrability of the mucus [17]. It is advisable to test the muciferous capacity of the crypts also after administration of exogenous oestradiol. If the score rises to 8 or more, stimulation has been successful.

Dajavan [5] observed abnormalities of the mucus in 50 to 60% of his patients. In 45% of these cases the quality, in the remaining cases the quantity of mucus was at fault. The favourable effect of oestradiol, indicated by the spinnbarkeit value and arborization, is qualitative as well as quantitative. Sperm penetration in this period is excellent.

Relative dysmucorrhoea responds well to oestradiol treatment. In case of absolute dysmucorrhoea, combination of oestradiol with antibiotics, eventually with electrocoagulation and cervical curettage is advocated, but the results are unconvincing [9, 24]. In penetration dysmucorrhoea which is presumably of immunological origin, the crossed test by the use of donor mucus and donor sperm may be informative, since the presence of antisperm-antibodies is apt to interfere with penetration and thus be responsible for infertility. Adrenocortical steroids are of little benefit, but a decline of the antibody-titres has been reported after regular long-term use of a protective sheet during coitus [22]. The necessity of intrauterine insemination may none the less arise [22].

The difference between the ovulation and conception rates after clomiphene therapy is due to the inhibitory effect of the drug on the cervix [29, 41]. An average ovulation rate of 70% versus a pregnancy rate of no more than 30% has been reported by the majority of authors. In one of our series the pregnancy rate was 34% [31]. The difference between the ovulation and pregnancy rates may be due to an inadequacy of the clomiphene-induced luteal phases [41], to an abnormal fallopian transport, or to the poor quality of the cervical mucus [13]. In this case the results may be considerably improved by combined clomiphene-oestradiol therapy [13, 33, 37]. According to the results of Lunenfeld and Insler [24], supported by our own observations, proper selection of the cases and combined therapy may narrow the gap between the two figures significantly.

## REFERENCES

1. Behrmann, S. J.: The immune response and infertility: Experimental evidence. In: Behrmann, S. J., Kistner, R. W. (eds): Progress in Infertility. Little, Brown and Co., Boston 1968, pp. 675–699.
2. Bernstein, D., Glezerman, M., Zejdel, L., Insler, V.: Quantitative study of the number and size of cervical crypts. In: Insler, V., Bettendorf, G. (eds): The Uterine Cervix in Reproduction. Georg Thieme Verlag, Stuttgart 1977, pp. 14–21.
3. Carlborg, L.: Determination of sperm migration rate in small samples of cervical mucus. Acta endocr. (Kbh.) **62,** 732–746 (1969).

4. Chretien, F. C.: Technical problems involved in scanning electron microscopic observation of cervical mucus framework. In: Insler, V., Bettendorf, G. (eds): The Uterine Cervix in Reproduction. Georg Thieme Verlag, Stuttgart 1977, pp. 44–52.
5. Davajan, V.: In vivo postcoital test. In: Hafez, E. S. E. (ed.): Techniques of Human Andrology. Elsevier-North-Holland Biomedical Press, Amsterdam 1977, pp. 373–378.
6. Davajan, V., Nakamura, R. M.: The cervical factor. In: Shermann, S. J., Kistner, R. W. (eds): Progress in Infertility. 2nd ed., Little Brown and Co., Boston, Mass. 1975, pp. 17–46.
7. Eichenbrenner, I., Insler, V., Serr, D. M., Salomy, M., Faktor, J.: The effect of clomiphene on vaginal cytology and cervical mucus. Harefuah **78,** 171–173 (1970).
8. Elstein, M., Daunter, B.: Trace elements in cervical mucus. In: Cervical mucus in human reproduction. Eds. M. Elstein, K. S. Moghissi and R. Borth. Scriptor, Copenhagen 1973 pp. 122–127.
9. Friedrich, E. R.: The normal morphology and ultrastructure of the cervix. In: Blandau, R. J., Moghissi, K. (eds): The Biology of the Cervix. The University of Chicago 1973, pp. 79–102.
10. Hulka, J. F., Omran, K. F.: The uterine cervix as a potential local antibody secretor. Amer. J. Obstet. Gynec. **104,** 440–442 (1969).
11. Insler, V.: Evaluation and treatment of cervical mucus diseases leading to infertility. In: Parke, D. V., Elstein, M. (eds): Mucus in Health and Disease. Plenum Publishing Co., New York 1977, pp. 477–486.
12. Insler, V., Melmed, H., Eichenbrenner, I., Serr, D. M., Lunenfeld, B.: The cervical score — a simple semi-quantitative method for monitoring of the menstrual cycle. Int. J. Gynec. Obstet. **10,** 223–228 (1972)
13. Insler, V., Zakut, H., Serr, D. M.: Cycle pattern and pregnancy rate following combined clomiphene-estrogen therapy. Obstet. and Gynec. **41,** 602–607 (1973)
14. Jaszczak, S., Hafez, E. S. E.: In vitro sperm penetration in cervical mucus. In: Hafez, E. S. E. (ed.): Techniques of Human Andrology. Elsevier-North-Holland Biomedical press, Amsterdam 1977, pp. 379.
15. Jaszczak, S., Hafez, E. S. E.: Physiopathology of sperm transport in the human female. In: Hafez, E. S. E., Thibault, C. G. (eds): The Biology of Spermatozoa. S. Karger, Basel 1975, pp. 250–256.
16. Jordan, J., Allen, J. M.: Ultrastructure of the cervical epithelium. In: Insler, V., Bettendorf, G. (eds): Uterine Cervix in Reproduction. Georg Thieme Verlag, Stuttgart 1977, pp. 21–35.
17. Kerin, J. F., Matthews, C. D., Svigos, J. M., Makin, A. E., Symons, R. G., Smeaton, T. C.: Linear and quantitative migration of stored sperm through cervical period. Fertil. and Steril. **27,** 1054–1069 (1976).
18. Kesserü, E.: In vivo sperm penetration and in vitro sperm migration tests. Fertil. and Steril. **24,** 584–591 (1973)
19. Kremer, J.: A simple sperm penetration test. Int. J. Fertil. **10,** 209–215 (1965).
20. Kremer, J.: The in vitro spermatozoal penetration test in fertility investigation. Thesis, University of Groningen (1968).
21. Kremer, J., Jager, S.: The sperm-cervical mucus contact test: a preliminary report. Fertil. and Steril. **27,** 335–340 (1976).
22. Kremer, J., Jager, S., Van Slochteren-Draaisma, T.: Treatment of infertility due to anti-sperm antibodies. In: Insler, V., Bettendorf, G. (eds). The Uterine Cervix In Reproduction. Thieme, Stuttgart 1977, p. 249.
23. Lamar, J., Shettles, L., Delfs, E.: Cyclic penetrability of human cervical mucus to spermatozoa in vitro. Amer. J. Physiol. **129,** 234–241 (1940).
24. Lunenfeld, B., Insler, V.: The cervical factor. In: Lunenfeld, B., Insler, V. (eds): Diagnosis and Treatment of Functional Infertility. Grosse Verlag, Berlin 1978, pp. 90–113.
25. MacDonald, R. R.: Cyclic changes in cervical mucus. J. Obstet. Gynaec. Brit. Cwlth. **76,** 1090–1099 (1969).
26. Miller, E. G., Jr., Kurzrok, R.: Biochemical studies of human semen: factors affecting migration of sperm through the cervix. Amer. J. Obstet. Gynec. **24,** 19 (1932)
27. Moghissi, K. S.: Sperm migration through the human cervix. In: Elstein, M., Moghissi, K. S., Borth, R. (eds): Cervical Mucus in Human Reproduction. Scriptor, Copenhagen 1973, pp. 128–152.
28. Moghissi, K. S.: Sperm migration through the human cervix. In: Insler, V., Bettendorf, G. (eds): The Uterine Cervix in Reproduction. Georg Thieme Verlag, Stuttgart 1977, p. 146.

29. Murray, M., Osmond-Clarke, F.: Pregnancy results following treatment with clomiphene citrate. J. Obstet. Gynaec. Brit. Cwlth **78,** 1108–1114 (1971).
30. Reichman, J., Insler, V., Serr, D. M.: A modified in vitro spermatozoal penetration test. Int. J. Fertil. **18,** 241–245 (1973).
31. Sas, M.: A functionalis meddőség. Orv. Hetil. **6,** 307–313 (1978).
32. Sas M., Farkas, M.: Pozitív családtervezés I. Az Orvostudomány Aktuális Problémái, 35. Medicina, Budapest, 1979.
33. Scharf, M. Graff, G., Kuzminski, T.: Quinestrol therapy in hypomucorrhoea due to clomiphene. Amer. J. Obstet. Gynec. **110,** 423–434 (1971).
34. Schumacher, G. F. B.: Soluble proteins in cervical secretions. In: Sherman, A. I., Charles, C. Thomas (eds): Pathways to Conception. Springfield, III., 1971, pp. 168–187.
35. Schwartz, R., Zinsser, H.: Some factors modifying sperm progression. Fertil. and Steril. **6,** 450–458 (1954).
36. Seegar-Jones, G., Maffezzoli, R. D., Rose, G. T., Kaplan, G.: Pathophysiology of reproductive failure after clomiphene-induced ovulation. Amer. J. Obstet. Gynec. **108,** 847–867 (1970)
37. Seki, M., Tajima, C., Maeda, H. R., Seki, K., Yoshihara, T.: Effect of quinestrol administered with clomiphene citrate on serum follicle-stimulating hormone and luteinizing hormone and other clinical findings. Amer. J. Obstet. Gynec. **116,** 388–396 (1973).
38. Shulman, S.: Immunologic barriers to fertility. Obstet. Gynec. Survey **27,** 553–606 (1972).
39. Sobrero, A. J.: Sperm migration in the human female. In: Campos da Paz, A., Hasegawa, T., Notake, Y., Hayashi, M. (eds): Human Reproduction. Igaki Shoin Ltd., Tokyo 1974, pp. 47–53.
40. Steiberger, E., Perloff, W. H.: In vitro penetration of cervical mucus by spermatozoa. Fertil. and Steril. **14,** 231–236 (1963).
41. Whitelaw, M. J., Kalma, C. F., Grams, L. R.: The significance of the high ovulation rate versus the low pregnancy rate with clomid. Amer. J. Obstet. Gynec. **107,** 865–877 (1970).
42. World Health Organization: In: Elstein, M., Moghissi, K. S., Borth, R. (eds): Cervical mucus in Human Reproduction. Scriptor, Copenhagen 1973.

*Acta Medica Hungarica, 41 (2—3), pp. 113—119 (1984)*

*Immunology*

# AUTOLOGOUS ROSETTE-FORMING CAPACITY OF T-LYMPHOCYTES IN HODGKIN'S DISEASE

Edit Bodolay, E. Berényi, Ildikó Sonkoly, G. Szegedi

THIRD DEPARTMENT OF MEDICINE, UNIVERSITY MEDICAL SCHOOL, DEBRECEN, HUNGARY

Received: January 26, 1983

Distribution of the T-lymphocyte subpopulation capable of binding own erythrocytes was examined in patients with Hodgkin's (H) disease. Parallel with the autologous rosette-forming cells (Tar-cells) the absolute lymphocyte count and the proportion of sheep-E-rosette forming T-cells were studied in various stages and histological types of H-disease. A considerable increase in the proportion of Tar-cells was found in untreated or recurrent H-disease, whereas in the periods of remission resulting from therapy no distinct differences between these figures and the mean values of the controls were demonstrable. In the generalized stage of the disease as well as in its mixed-cell and lymphocyte depletion types of poor prognosis, the proportion of Tar-cells was increased. The absolute lymphocyte count and the proportion of T-cells showed a significant fall with the increase of Tar-cells. It is assumed on the grounds of the functional properties of T-cells that the increase in the proportion of Tar-cells in H-disease may be interpreted as a sign of suppressor hyperactivity.

**Keywords:** autologous rosette-forming cells, regulator mechanism, helper-suppressorbalance, clinical stage, histological type, Hodgkin's disease

## Introduction

In Hodgkin's disease (H) the delayed type immune reactivity is mainly affected. Blast transformation of lymphocytes in response to various mitogens is depressed, homograft-rejection is delayed, the susceptibility to bacterial and viral infections is high [1, 2, 5, 10, 12, 13, 16, 19, 23, 36, 38]. According to Moretta et al. [27], Romagnani et al. [32] and others [3, 23, 37, 39], the proportion of the individual T-lymphocyte subpopulations is also altered, and an increase in the T-cell population of suppressor type is assumed to contribute to the impairment of immune reactivity.

The present study has been concerned with the number and proportion of autologous erythrocyte binding T-cells in H-disease. The character of these T-cells is like of young T-precursor cells migrating from the thymus into the blood stream. They have not yet developed surface IgG or IgM binding Fc-receptor structures. They are vulnerable to steroid hormones in vitro and in

Send offprint requests to Dr. Edit Bodolay, Third Department of Medicine, University Medical School, H-4004 Debrecen, P. O. Box 3, Hungary

vivo alike. Approximately 20% of the Tar-cells bind peanut-agglutinin (PNA), as a sign that the lymphocytes of this group are still undifferentiated. Their differentiation in the helper or suppressor direction proceeds in vitro under the effect of thymic hormones or of concanavalin-A [29]. On the evidence of studies with monoclonal antibodies, the Tar-cells are heterogeneous, the majority being already identifiable with the surface markers Okt 4 (helper) or Okt 8 (suppressor) [15]. According to Palacios et al. [30] and Heijnen et al. [15], this lymphocyte subpopulation plays an important part in the production and maintenance of the helper-suppressor balance. The scarcity of information on the numerical distribution and role of the Tar-cells in the various human malignant diseases has prompted us to examine the questions how the number of Tar-cells, compared with the mean values of normal controls, was affected in H-disease, whether it was related to the extent or grade of the disease and in what manner it responded to therapy. The proportion of sheep E-rosette-forming T-cells was also examined parallel with that of the Tar-cells.

## Patients and methods

Thirtyfour patients with H-disease were studied. Their average age was 48.5 years, the male to female ratio was 15:19. Clinical staging was based on the Ann-Arbor criteria [9]. Five of the patients had recently diagnosed untreated H-disease, 29 patients had undergone chemotherapy or/and radiotherapy. Among the five untreated patients in 4 the disease was in clinical stage III and one patient stage IV. At the time of the study, of the treated patients 2 were in clinical stage I, 9 in clinical stage II, 12 in clinical stage III and 6 in clinical stage IV. The histological type corresponded to the lymphoproliferative form (LP) in 8, to nodular sclerosis (NS) in 8, to the mixed cell type (MC) in 15 and to the lymphocyte depletion (LD) type in 3 cases [25]. Of the treated patients 12 were in an active, 17 in an inactive period of the disease. In addition to the still untreated and to the recurrent cases those with incomplete remission were also regarded as active. Twentyone patients were subjected to chemotherapy or $^{60}$Co-irradiation in mantle or inverted Y field, and 4 had combined therapy. The interval between completion of treatment and the studies averaged 6.5 months (1 to 25 months). The data of 30 normal subjects, of 35.5 years average age served as control.

For lymphocyte separation from venous blood the method of Böyum [6] was used. The E-rosette test was performed according to Jondal et al. [17].

*Autologous rosette-test*

After the last washing the lymphocytes were taken up in Ca and Mg free Hank's solution and the cell-count was adjusted to $2 \times 10^7$/ml. The erythrocytes were washed in Ca and Mg free Hank's solution, and a cell suspension of $3 \times 10^8$/ml was prepared. To a mixture of 0.1 ml lymphocytes ($2 \times 10^6$) and of 0.1 ml autologous erythrocytes ($3 \times 10^7$) calf serum exhausted with human erythrocytes of group AB was added in a small test-tube. The mixture was centrifuged and incubated at +4 °C for 24 h.

Evaluation: 2 min before resuspension the lymphocytes were stained with 0.2% toluidine blue in Ca and Mg free Hank's solution. The number of lymphocytes attaching a minimum of three human erythrocytes was referred to 200 lymphocytes.

For statistical analysis Student's *t* test was used. The results are expressed as mean ± SD.

## Results

The absolute lymphocyte count was higher in the normal controls than the mean for the patients with treated or untreated H-disease ($P < 0.001$) (Table I) and if the comparison was based on the stage of progression, the

**Table I**

*Proportion of $T_t$ and Tar-cells in the peripheral blood of patients with Hodgkin's disease and of normal subjects* (mean ± S.D.)

| | Absolute lymphocyte count | T total rosettes per cent | f T autologous rosettes per cent |
|---|---|---|---|
| Control group n = 30 | 2823.5 ± 310 | 69.2 ± 5.3 | 20.4 ± 3.1 |
| Patients with untreated Hodgkin's disease n = 5 | 1660.4 ± 207** | 46.2 ± 3.8** | 28.6 ± 2.2** |
| Patients with treated Hodgkin's disease n = 29 | 1417.6 ± 630** | 50.2 ± 5.9* | 23.6 ± 1.8* |
| Active period n = 12 | 1150.5 ± 420** | 39.2 ± 4.7** | 28.2 ± 2.3** |
| Inactive period n = 17 | 1695.6 ± 250** | 56.8 ± 3.2 | 19.6 ± 2.0 |

* = $P < 0.01$
** = $P < 0.001$
n = number of patients

difference was still more marked. In the MC and LD types of poor prognosis the absolute lymphocyte counts were lower than in the LP and NS forms of the disease. The number of E-rosette forming T-cells also failed to attain the mean value for the control group in the treated and untreated groups alike ($P < 0.001$). In the active periods of the disease the number of T-lymphocytes was lower than in the periods of remission. In the generalized stage, as well as in the MC and LD types, the number of T-cells identifiable on the ground of their rosette-forming capacity was also lower (Table II).

In untreated H-disease the number of Tar-cells was significantly higher than in the group of normal controls ($P < 0.001$). In the patients with treated H-disease the number of Tar-cells was increased, but not significantly so. An increase in the proportion of Tar-cells was demonstrable in treated, active cases ($P < 0.001$) as also in the MC and LD types ($P < 0.01$). In the 4 inactive patients who had undergone radiotherapy 4 to 12 months earlier, the number of Tar-cells was considerably reduced (14% ± 2.5).

**Table II**

*Proportion of $T_t$ and Tar-lymphocytes in patients with treated Hodgkin's disease, according to clinical stage and histological type* (mean±S.D.)

| | Absolute lymphocyte count | T total rosettes per cent | T autologous rosettes per cent |
|---|---|---|---|
| Control group | | | |
| n = 30 | 2823.5 ± 310 | 69.2 ± 5.3 | 20.4 ± 3.1 |
| Patients with Hodgkin's disease | | | |
| Clinical stage | | | |
| I. n = 2 | 2078.0 ± 150 | 60.5 ± 3.2 | 19.5 ± 3.9 |
| II. n = 10 | 1660.8 ± 180** | 49.4 ± 3.8** | 20.5 ± 2.9 |
| III. n = 11 | 1281.6 ± 230** | 50.3 ± 2.4** .. | 24.0 ± 3.4 |
| IV. n = 6 | 1448.0 ± 450** | 40.3 ± 5.3** | 28.2 ± 1.2** |
| According to histological type | | | |
| LP — NS n = 11 | 1509.1 ± 120** | 51.5 ± 5.3** | 19.6 ± 3.4 |
| MC — LD n = 18 | 1204.9 ± 230** | 47.4 ± 4.9** | 26.9 ± 4.2* |

* = $P < 0.01$
** = $P < 0.001$

## Discussion

Surface marker studies and functional methods have revealed a deficiency of cellular immunity in H-disease, but contradictory reports have also appeared [1, 2, 23]. The humoral immune reactivity remains unaffected until the advanced stage of the disease [35]. There are various findings pointing to the prevalence of a suppressor effect. Impairment of lymphocyte transformation in response to phytohaemagglutinin (PHA) is also attributed to a suppressor cell hyperactivity, for which partly an increase of the prostaglandin-producing monocyte population, partly the T-suppressor cells [3, 4] are incriminated. The results of Moretta et al. [27] and Romagnani et al. [32] indicate that in H-disease the $T_G/T_M$-lymphocyte quotient is increased, in other words there is prevalence of T-lymphocytes carrying IgG-Fc-receptors. In patients with untreated H-disease the number of the T-lymphocytes of suppressor type provided with histamine receptors is increased, but treatment results in a fall of the number of the $T_G$-cells, as well as of the T-cells with histamine receptors, to approximately normal values [3, 4, 27, 32].

In the peripheral blood of normal subjects the number of Tar-cells, that is of the cells which are assumed to play a regulatory role in the maintenance of the helper-suppressor-balance, ranges between 20 and 25% [30, 31]. Accord-

ing to the findings of Fournier and Charreire [11], in response to concanavalin-A pretreatment the Tar-cells increased in number and assumed the functional characters of lymphocytes of effector suppressor type. Palacios et al. [30] found purified Tar-cells inhibitory to immunglobulin synthesis induced by pokeweed mitogen (PWM). According to Sakane et al. [34] concanavalin-A induced Tar-lymphocytes proved inhibitory also to PWM induced B-cell proliferation. In autologous mixed lymphocyte cultures an increased proportion of Tar-cells capable of binding the monoclonal serum Okt 8 was found by Kumagai et al. [20]. It has been demonstrated by these studies that Tar-cells maturate to suppressor effector cells in response to antigenic or mitogenic stimuli.

The data concerning the number of Tar-cells in human malignant disease are inconsistent [22]. Sandilands et al. [33] have observed increased figures in a group of melanoma patients while Caraux et al. [8] found low figures in a material of more than 100 patients with solid tumour. The Tar-cells in H-disease have been studied by Lang et al. [21]; no significant difference in the autologous rosette-forming capacity between patient and control lymphocytes has been found by these authors in 24 untreated cases of H-disease.

The present study shows that, in accordance with earlier evidence, the sheep-E-binding spontaneous E-rosette forming capacity of the lymphocytes is deficient in H-disease, a finding sufficient in itself to be suggestive of functional abnormalities of T-cells. In the treated cases being in remission, the number of Tar-cells was not significantly different from the mean values of normal controls. On the other hand, in the untreated and in the recurrent cases the proportion of the Tar-cells was considerably augmented. The increase in their proportion was the more remarkable, as in the same group of patients the absolute lymphocyte counts as well as the proportion of T-cells were reduced. In the patients who had undergone radiotherapy, the autologous rosette-forming capacity of the T-lymphocytes was deficient 6 to 12 months after therapy. This would seem to indicate that the Tar-cells represent a highly radiosensitive lymphocyte population.

In earlier studies [3, 4] we have found, similarly to other workers, a general increase in the number of T-cells of suppressor type in H-disease, depending on the activity and other features of the process. In the present series an increase in the number and proportion of Tar-cells was demonstrable. These changes are indicative of the severity of the process, on the one hand, and may help to monitor its therapy, on the other.

## Acknowledgement

This work was supported by the National Foundation for Cancer Research, Bethesda, U.S.A.

## REFERENCES


1. Aisenberg, A. C.: Studies on delayed hypersensitivity in Hodgkin's disease. J. clin. Invest. **41,** 1964–1970 (1962).
2. Andersen, E.: Depletion of thymus-dependent lymphocytes in Hodgkin's disease. Scand. J. Haemat. **12,** 263–269 (1974).
3. Berényi, E., Sonkoly, I., Kávai, M., Szegedi, Gy.: Szuppresszor T sejt markerek vizsgálata Hodgkin-kóros betegek perifériás vérében. Magy. Belorv. Arch. **33,** 309–314 (1980).
4. Berényi, E., Pálóczi, K., Sonkoly, I., Pálkövi, E., Szegedi, Gy.: Marker vizsgálatok Hodgkin-kóros betegek perifériás lymphocytáin. Magy. Onkol. **24,** 7–16 (1980).
5. Bobrove, A. M., Fuks, Z., Strober, S., Kaplan, H. S.: Quantitation of T and B lymphocytes and cellular immune function in Hodgkin's disease. Cancer **36,** 169–179 (1975).
6. Böyum, A.: Isolation of mononuclear cells and granulocytes from human blood. Scand. J. Lab. Invest. **21,** 77–89 (1968).
7. Bukowski, R. N., Noguchi, S., Hewlett, J. S., Deodhar, S.: Lymphocyte subpopulations in Hodgkin's disease. Amer. J. clin. Path. **65,** 31–39 (1976)
8. Caraux, J., Thierry, C., Serrou, B.: Human autologous rosettes II. Prognostic significance of variations in autologous rosette-forming cells in the peripheral blood of cancer patients. J. nat. Cancer Inst. **63,** 593–597 (1979).
9. Carbone, P. P., Kaplan, S., Mushoff, K., Smitters, D. W., Tubiana, M.: Report of the committee on Hodgkin's disease staging classification. Cancer Res. **31,** 1860–1861 (1971).
10. Casarza, A. R., Duvall, C. P., Carbone, P. P.: Summary of infectious complications occurring in patients with Hodgkin's disease. Cancer Res. **26,** 1290–1296 (1966).
11. Fournier, C., Charreire, J.: Activation of a human cell subpopulation bearing receptors for autologous erythrocytes by concanavalin A. J. Immunol. **121,** 711–717 (1978).
12. Galj-Peczalska, K. J., Bloomfield, C. D., Sosin, H., Kersey, J. H.: B and T lymphocytes in Hodgkin's disease; analysis at diagnosis and following therapy. Clin. exp. Immunol. **23,** 47–55 (1976).
13. Goffinet, D. R., Glatstein, E. J., Merigan, T. C.: Herpes zoster varicella infections and lymphoma. Ann. intern. Med. **76,** 235–240 (1972).
14. Goodwin, J. S., Messner, R. P., Bankhurst, A. D., Peake, G. T., Saiki, J. H., Williams, R. C.: Prostaglandin-producing suppressor cells in Hodgkin's disease. New Engl. J. Med. **297,** 963–970 (1977) .
15. Heijnen, C. J., Pot, K. H., Kater, L., Kluin-Nelemans, H. C., Uytdehaag, F., Ballieux, R. E.: Functional analysis of the defective T cell regulation of the antigen-specific PFC response in SLE patients- differentiation of suppressor precursor cells to suppressor effector cells. Clin. exp. Immunol. **47,** 359–367 (1982).
16. Hillinger, S. M., Herzig, G. P.: Impaired cell-mediated immunity in Hodgkin's disease mediated by suppressor lymphocytes and monocytes. J. clin. Invest. **61,** 1620–1627 (1978).
17. Jondal, M., Holm, G., Wigzell, H.: Surface markers on human T and B lymphocytes I. A large population of lymphocytes forming nonimmune rosettes with sheep red blood cells. J. exp. Med. **136,** 207–215 (1972).
18. Kaplan, J.: Human T lymphocytes from rosettes with autologous and allogenic human red blood cells. Clin. Immun. Immunopath. **3,** 471–475 (1975).
19. Kelly, W. D., Good, R. A., Varco, R. L.: Anergy and skin homograft survival in Hodgkin's disease. Surg. Gynec. Obstet. **107,** 565–570 (1958).
20. Kumagai, S., Suginoshita, T., Green, I.: Autologous rosette-forming T cells (ARFT) regulate responses of T cells. Hum. Immunol. **5,** 35–48 (1982).
21. Lang, J. M., Hasselmann, J. P., Giron, C., Bigel, P., Bergerat, J. P., Bernard, C., Boilletot, A., Oberling, F., Mayer, S.: Comparative study of autologous, total E and "active" E-rosette-forming T lymphocytes in untreated Hodgkin's disease. Cancer Immun. Immunother. **10,** 239–242 (1981).
22. Lang, J. M., Hasselmann, J. P., Roeslin, N., Mayer, S., Witz, J. P.: Comparative study of autologous and "active" rosette forming T lymphocytes in untreated patients with non-metastatic squamosus-cell lung carcinoma. Cancer Immun. Immunother. **8,** 45–47 (1980).
23. Levy, R. Kaplan, H. S.: Impaired lymphocyte function in untreated Hodgkin's disease. New Engl. J. Med. **289,** 181–186 (1974).
24. London, J., Berrih, S. Bach, J. F.: Peanut agglutinin I. A new tool for studying T lymphocyte subpopulations. Immunology **121,** 438–443 (1978).
25. Lukes, R. J., Buttler, J. J.: The pathology and nomenclature of Hodgkin's disease. Cancer Res. **26,** 1063–1074 (1966).

26. Matchett, K. M., Huang, A. T., Kremer, W. B.: Impaired lymphocyte transformation in Hodgkin's disease: evidence for depletion of circulating T-lymphocytes. J. clin., Invest. **52,** 1908–1917 (1973).
27. Moretta, L., Mingari, M. C., Moretta, A.: Human T cell subpopulations in normal and pathologic conditions. Immunol. Rev. **45,** 163–193 (1979).
28. Palacios, R., Llorente, L., Alarcon-Segovia, D., Arguelles, A. R., Jouanen, E.: Autologous rosette-forming T cells as the responding cells in human autologous mixed lymphocytes reaction. J. clin. Invest. **65,** 1527–1530 (1980).
29. Palacios, R.: Role of the autologous rosette-forming T cells in the concanavalin A induced suppressor cell function. Cell. Immunol. **61,** 273–279 (1981).
30. Palacios, R., Alarcon-Segovia, D.: Human post thymic precursor cells in health and disease. Scand. J. Immunol. **13,** 499–502 (1981).
31. Palacios, R., Alarcon-Segovia, D., Llorente, L., Ruiz-Arguelles, A., Diaz-Jouanen, E.: Human post thymic precursor cells in health and disease I. Characterisation of the autologous rosette-forming T cells as post thymic precursors. Immunology **42,** 127–135 (1981).
32. Romagnani, S., Maggi, E., Biagiotti, R., Gindizi, M. G., Amadori, A., Ricci, M.: Altered proportion of T-cell subpopulations in patients with Hodgkin's disease. Scand. J. Immunol. **7,** 511–522 (1978).
33. Sandilands, G. P., Gray, K. G., Conney, A. E., Browning, J. D., Anderson, J. R.: Autorosette formation by human thymocytes and lymphocytes. Lancet **1,** 27–32 (1974).
34. Sakane, T., Honda, M., Taniguchi, Y., Kotani, H.: Separation of concanavalin A-induced human suppressor and helper T cells by autologous erythrocyte rosette technique. J. clin. Invest. **68,** 447-453 (1981).
35. Schier, W. W., Roth, A., Ostroff, G., Schrift, M. H.: Hodgkin's disease and immunity. Amer. J. Med. **20,** 94–99 (1956).
36. Sokal, J. E., Primikirios, N.: The delayed skin test response in Hodgkin's disease and lymphosarcoma: effect of disease activity. Cancer **14,** 597–607 (1961).
37. Twomey, J. J., Laughter, A. H., Farrow, S.: Hodgkin's disease: an immundepleting and immunsuppressive disorder. J. clin. Invest. **56,** 467–475 (1975).
38. Winkelstein, A., Mikulla, J. M., Sartiano, G. P., Ellis, L. D.: Cellular immunity in Hodgkin's disease: comparison of cutaneous reactivity and lymphoproliferative responses to phytohaemagglutinin. Cancer **34,** 549–553 (1974).
39. Zembala, M., Mytar, B., Popiela, T., Asherson, G. L.: Depressed in vitro peripheral blood lymphocyte response to mitogens in cancer patients: the role of suppressor cells. Int. J. Cancer **19,** 605–613 (1977).

*Acta Medica Hungarica, 41 (2—3), pp. 121—127 (1984)*

# SURFACE MARKERS OF BLOOD LYMPHOCYTES IN BRONCHIAL CARCINOMA

Edit Bodolay, G. Szabó, I. Juhász[1], Zsuzsanna Komoróczy, Andrea Diczházy

THIRD DEPARTMENT OF MEDICINE AND [1]SECOND DEPARTMENT OF SURGERY, UNIVERSITY MEDICAL SCHOOL, DEBRECEN, HUNGARY

Received: December 22, 1982

The proportion of autologous rosette-forming, i.e. autoerythrocyte-binding, T-lymphocytes, was studied in 34 patients with untreated, operable bronchial carcinoma. The number of autorosettes in patients with bronchial carcinoma was considerably below the mean value for the normal control group. In patients with metastatic involvement of the regional lymph nodes at the time of surgery the reduction in the number of autorosettes was still more marked. On the 10th postoperative day after resection of the tumour and in case of remission five months after surgery, the number of autorosettes showed a significant rise approximating the normal value. The distribution of sheep-E-rosette-forming and of the "active-early" T-lymphocytes was also studied. On the evidence of the results, the number of autorosette-forming lymphocytes lends itself to a follow-up of bronchial carcinoma by early demonstration of remissions or recurrences.

**Keywords:** autologous rosette-forming cells, regulatory function, untreated bronchial carcinoma

## Introduction

In bronchial carcinoma cellular immune reactivity is generally affected and declines with progress of the disease. This is reflected by the negativity of intradermal tests [18] and by a depression of lymphocyte transformation in response to mitogens [12, 27].

Among the T-lymphocytes 20–25% has the capacity of forming rosettes, in addition to sheep erythrocytes, also with autologous erythrocytes (Tar = T autologous rosette) [4, 5, 6, 9, 13, 14, 16]. According to present knowledge, the T-lymphocytes capable of forming rosettes with autoerythrocytes represent a precursor cell population a part of which has already attained the stage of reacting with the monoclonal antibodies Okt 4–8, i.e. they represent helper or suppressor cells, whereas the other, substantial part belongs to the functionally noncommitted T-precursor cells. The Tar-cells lack a surface-IgG- or IgM-binding Fc-receptor structure. They are vulnerable to steroid hormones in vivo and in vitro alike, and are resistant to theophylline. They represent

Send offprint requests to Dr. Edit Bodolay, Third Department of Medicine, University Medical School, H-4004 Debrecen, P.O. Box 3, Hungary

a lymphocyte population which plays an important part in the maintenance of normal feed-back inhibition [19, 23, 24, 25, 26]. In the present study the proportion of the autorosette-forming population was studied in patients with bronchial carcinoma and compared with the mean values of a normal control group. It was examined how their proportion is affected by surgical removal of the tumour and whether their study was suitable for the follow-up of the clinical situation. The active and total lymphocyte counts have also been determined and correlated to the number of Tar-cells.

## Patients and methods

Thirtyfour patients with operable bronchial carcinoma, aged 41–86 (average 56.5) years were studied. The male-to-female ratio was 32 : 2.

None of the patients had received any therapy before. The surgical specimens were subjected to microscopic study in all of the cases. Clinical staging was based on the TNM-classification. Of the patients, 14 were in stage T1, 16 in stage T2, 4, in stage T3. At surgery 14 patients displayed microscopically demonstrable deposits in the regional lymph nodes. (The histological studies were performed at the Institute of Pathology, University Debrecen).

The patients were studied for rosette-forming cells on the day before surgery; 20 patients were reexamined on the 10th postoperative day, 15 patients were available for follow-up studies five months after surgery. Thirty normal subjects served as controls.

*Lymphocyte separation.* Heparinized venous blood was separated on Ficoll-gradient, according to Böyum [2].

For the E-rosette test the method described by Yu and Jondal was used [13, 31]. To 0.1 ml lymphocyte suspension ($10^7$ cells/ml) a suspension of washed sheep red cells ($2 \times 10^8$ cells/ml) of the same volume was added. After incubation at 37 °C for five min the mixture was centrifuged at 200 *g* for 10 min. Directly after resuspension the number of early, "active", rosettes, and after incubation at +4 °C overnight the total number of E-rosettes was determined, the lymphocytes binding a minimum of three sheep red cells being counted among 200 lymphocytes.

*Autologous rosette-test.* The lymphocytes separated on Ficoll-gradient were taken up after the last washing in Ca- and Mg-free Hank's solution (pH 7.4), and the cell count was adjusted to $2 \times 10^7$ ml.

The autologous erythrocytes were washed in Ca- and Mg-free Hank's solution and made up to a cell suspension of $3 \times 10^8$ ml. To a mixture of 0.1 ml lymphocytes ($2 \times 10^6$), 0.1 ml autologous erythrocytes ($3 \times 10^7$) and 0.1 ml calf serum exhausted with human erythrocytes of group AB was added in a test tube, centrifuged at room temperature at 200 *g* for five min and incubated at +4 °C for 24 h.

Evaluation: two min before resuspension the lymphocytes were stained with a drop of 0.2% toluidine blue in Ca-and Mg-free Hank's solution. The lymphocytes attaching a minimum of three human erythrocytes per 200 lymphocytes were counted under a coverglass.

At the same time the absolute lymphocyte count per $mm^3$ was also determined, on the basis of the leukocyte count and the percentage of lymphocytes.

For statistical evaluation Student's *t* test was used. The results are expressed as mean $\pm$ S.D.

## Results

No significant differences in the absolute lymphocyte count were demonstrable between the patients with bronchial carcinoma and the normal controls (normal controls $2838.7 \pm 474.4$; bronchial carcinoma patients $2495.1 \pm 995.8$).

Before surgery, the active, total and autologous rosettes were significantly reduced in number (Table I).

**Table I**

*Changes in the rosette surface markers in patients with bronchial carcinoma*

| | Control n = 30 | Bronchial carcinoma n = 34 | Significance |
|---|---|---|---|
| T active | 22.0 ± 3.5 | 14.6 ± 6.3 | $P < 0.001$ |
| T total | 69.2 ± 5.2 | 46.2 ± 11.6 | $P < 0.001$ |
| T autologous | 20.3 ± 2.8 | 10.7 ± 6.3 | $P < 0.001$ |

mean ± S.D.
n = number of patients

In the patients with metastatic involvement of the regional lymph-nodes at the time of surgery the fall in the number of autorosettes was still more marked, whereas in the number of T-active and T-total rosettes there was practically no difference between the metastatic and non-metastatic group (Table II).

**Table II**

*Changes in the rosette surface markers in patients with deposit-forming bronchial carcinoma*

| | Deposits n = 14 | No deposits n = 20 | Significance |
|---|---|---|---|
| T active | 14.4 ± 7.7 | 14.8 ± 4.0 | NS |
| T total | 45.5 ± 12.9 | 47.1 ± 9.5 | NS |
| T autologous | 8.0 ± 5.6 | 12.0 ± 4.2 | $P < 0.001$ |

mean ± S.D.
n = number of patients

Reexamination of 20 patients on the 10th postoperative day showed a significant increase in the number of autologous rosettes, whereas the active and total T-cells remained practically unchanged in number. Five months after successful surgical resection we were able to repeat the tests in 15 cases of these, 12 patients were in complete remission, while 3 patients had low-grade fever or deposits. An increase in the active and total T-cell counts was found, but it was significant for the total T-cell count only. On the other hand, the number of autologous rosettes had attained values approximating those of the normal controls (T-active: from 14.6% ± 6.3 to 16.2% ± 4.2; T-total:

**Table III**

*Changes in the rosette surface markers in postoperative periods*

| | Before operation n = 34 | | 10 days after operation n = 20 | 5 months after operation n = 15 | |
|---|---|---|---|---|---|
| T active | 14.6 ± 6.3 | NS | 15.2 ± 4.2 | 16.2 ± 4.2 | NS* |
| T total | 46.2 ± 11.6 | NS | 48.9 ± 10.3 | 58.1 ± 9.8 | $P < 0.01$ |
| T autologous | 10.7 ± 6.3 | $P < 0.01$ | 16.6 ± 3.5 | 19.0 ± 9.9 | $P < 0.001$ |

mean ± S.D.
n = number of patients

* significance referred to the preoperative value

from 46.2% ± 11.6 to 58.1% ± 9.8; T-autologous: from 10.7% ± 6.3 to 19.0% ± 9.9) (Table III). In the three patients with relapse the number of autologous rosettes was as low as before surgery 14%, 10% and 8%, respectively.

## Discussion

The peripheral lymphocyte count is usually low in the case of malignant tumours, including bronchial carcinoma [1, 6]; the reduction involves primarily the T-cells and is regarded as an adverse prognostic sign, as pointed out by Gross et al. [10] and Anthony et al. [1].

The function of the remaining T-cells is also affected, as reflected by a poor responsiveness to non-specific mitogens and in the negativity of intradermal tests [12, 18, 27]. According to the findings of Garcia and Valverde [8], and of Neubert et al. [22], in patients with bronchial carcinoma the reduced T-lymphocyte count demonstrated by the E-rosette test provides no reliable information on the clinical condition, owing to a considerable overlap between the values found in the tumour patients and in normal controls. Within the T-cell population the subgroup of lymphocytes capable of binding autologous erythrocytes has been receiving increasing attention in recent years [24, 25, 26]. In the blood of normal subjects the proportion of T-lymphocytes capable of recognizing autologous erythrocytes ranges between 20 and 25%, depending on the method used. A part of this population binds peanut agglutinin (PNA), as a sign that these cells have not yet attained full maturation, but are already committed to a helper or suppressor function. They are regarded by Palacios et al. [25] as T-cells of regulatory function playing an important part in the maintenance of the normal feedback inhibition and in cytotoxic cell functions.

Published evidence relating to the number of rosette-forming lympho-

cytes in malignant disease is scanty and inconsistent. According to the observations of Caraux et al. [3] of 112 patients with various tumours, the number of rosette-forming cells was considerably lower in patients with malignant disease than in normal subjects. Having followed up the autorosette-forming population for 16 months, these authors found that its number remained below normal even during remission after chemotherapy or irradiation. The autorosette-forming population a few months before the clinical relapses underwent different changes. In a group an initial rise of the value was followed by a sharp fall before relapse. In other patients number of Tar-cells declined gradually and in a third group it persisted at the normal level. In opposition to these authors, Lang et al. [17], in a material of 21 patients with bronchial carcinoma, found no difference in the number of Tar-cells between those of the patients and of normal controls.

No positive correlations were found between the number of rosette-forming T-cells and the duration or progress of the tumour, nor did the number of early-active rosette-forming cells correlate with that of the Tar-cells [17].

The data in the literature fail to warrant the conclusion that a reduction of the number of circulating T-cells, or of active rosette-forming cells was typical of a malignant process, although lymphopenia is certainly an adverse prognostic sign. Fudenberg et al. [6] and Kerman et al. [15] found the percentage of active T-cells normal or even increased in patients with untreated solid tumour. The number of active or total T-cells has therefore no reliable predictive value and is unsuited for following up of the process.

Our earlier results indicate that in patients with bronchial carcinoma the number of total lymphocytes and within this population that of T-cells identifiable with alpha-naphthyl-acetate esterase (ANAE), is not significantly reduced. On the other hand, the enzyme reaction has changed its type: the proportion of T-cells marked by a granular enzyme activity has increased. On the ground of the results of Gross et al. [10] and Moretta et al. [21] the increase may be connected with functional alterations of the T-cells. According to the present results, in malignant disease the number of T-cells forming spontaneous E-rosettes with sheep red cells is considerably reduced, which in itself is indicative of some functional disturbance.

The number of autorosette-forming T-cells, regarded as precursor T-cells, was also reduced in the present material and their reduction was found to correlate with the clinical course and with the presence of deposits.

Palacios et al. assumed that the autologous rosette-forming cells are precursors of natural killer (NK) cells: a restricted cytotoxicity might thus well account for the progression of the tumour [25]. The views concerning the NK-activity of the autorosette-forming lymphocytes are, however, divided. According to the findings of Rucheton et al. [29], the autologous rosette-forming lymphocytes have no NK-activity.

As it is known, the IC-level is high in malignant disease, but it declines after resection of the tumour and rises again in the case of a relapse [7, 11, 28].

The IC have been shown to inhibit E-rosette formation and binding of IgG-sensitized bovine red cells to the Fc-receptors in SLE [21]. IC might well be capable of altering other membrane structures as well, including those required for the binding of own erythrocytes, thus accounting for the reduction in the number of autologous rosettes. The effect of circulating serum factors and of tumour extracts on autologous rosette formation offer further lines of study.

According to the present findings, bronchial carcinoma is certainly associated with a reduction in the number of E-rosette-forming T-cells. For assessment of the clinical course, the number of the autologous rosette-forming T-cells may offer information. In view of its simplicity and of the predictive value of its results as regards remissions or relapses, the method is likely to provide a convenient tool for follow-up studies.

**Acknowledgement:**

This work was supported by the National Foundation for Cancer Research, Bethesda, U.S.A.

REFERENCES

1. Anthony, H. M., Kirk, J. A., Madsen, K. E., Mason, M. K., Templeman, G. H.: E and EAC rosetting lymphocytes in patients with carcinoma of bronchus. I. Some parameters of the test and of its prognostic significance. Clin. exp. Immunol. **20,** 29–40 (1975).
2. Böyum, A.: Isolation of mononuclear cells and granulocytes from human blood. Scand. J. Lab. Invest. **21,** 77–89 (1968).
3. Caraux, J., Thierry, C., Serrou, B.: Human autologous rosettes. II. Prognostic significance of variations in autologous rosette-forming cells in the peripheral blood of cancer patients. J. nat. Cancer Inst. **63,** 593–597 (1979).
4. Caraux, J., Serrou, B.: The binding of human lymphocytes to autologous erythrocytes. Biomedicine **29,** 315–318 (1978).
5. Caraux, J., Thierry, C., Esteve, C.: Human autologous rosettes. I. Mechanism of binding of autologous erythrocytes by T cells. Cell. Immunol. **45,** 36–48 (1979).
6. Fudenberg, H. H., Wybran, J., Robbins, D.: T rosette forming cells, cellular immunity and cancer. New Engl. J. Med. **292,** 475–476 (1975).
7. Füst, G., Fekete, B., Angyal, I., Jakab, Á., Pál, A., Merétey, K., Falus, A., Török, K., Szegedi, Gy., Kávai, M., Puskás, É., Csécsi-Nagy, M., Szabó, T., Lenkey, Á., Misz, M.: Evaluation of different methods for detecting circulating immune complexes, studies in patients with lung cancer. J. Immunol. Methods **46,** 259–276 (1981).
8. Garcia, S. F., Valverde, V.: Lymphocytes subpopulations in cancer patients. Relationship with type and clinical stage. Bull. Cancer **68,** 32–39 (1981).
9. Gluckman, J. C., Montambault, P.: Spontaneous autorosette forming cells in man. A marker for a subset population of T lymphocytes? Clin. exp. Immunol. **22,** 302–310 (1975).
10. Gross, L. R., Latty, A., Williams, E. A., Newberne, P. M.: Abnormal spontaneous rosette formation and rosette inhibition in lung carcinoma. New Engl. J. Med. **292,** 439–441 (1975).
11. Guy, K., Di Mario, U., Irvine, W. J., Hunter, A. M., Handley, A., Horne, N. W.: Circulating immune complexes and autoantibodies in lung cancer. Brit. J. Cancer **43,** 276–283 (1981).
12. Jerrels, T. R., Dean, J. H., Herberman, R. B.; Relationship between T-lymphocyte levels and lymphoproliferative responses to mitogens and alloantigens in lung cancer patients. Int. J. Cancer **21,** 282–290 (1978).

13. Jondal, M., Holm, G., Wigzell, H.: Surface markers on human T and B lymphocytes. I. A large population of lymphocytes forming non immune rosettes with sheep red cells. J. exp. Med. **136,** 207–215 (1972).
14. Kaplan, J.: Human T lymphocytes form rosettes with autologous and allogenic human red blood cells. Clin. Immun. Immunopath. **3,** 471–475 (1975).
15. Kerman, R. H., Smith, R., Stefani, S. S., Ezdinli, E.: Active T rosette forming cells in the peripheral blood of cancer patients. Cancer Res. **36,** 3274–3278 (1976).
16. Lambermont, M., Wybran, J., Govaerts, A.: Human autologous and allogenic rosettes. J. Immunol. Methods **15,** 157–162 (1977).
17. Lang, J. M., Hasselmann, J. P., Roeslin, N., Mayer, S., Witz, J. P.: Comparative study of autologous and "active" rosette-forming T lymphocytes in untreated patients with non metastatic squamosus-cell lung carcinoma. Cancer Immun. Immunother. **8,** 45–47 (1980).
18. Lee, Y. T. N., Sparks, F. C., Eilber, F. R., Morton, D. L.: Delayed cutaneous hypersensitivity and peripheral lymphocyte counts in patients with advanced cancer. Cancer (Philad.) **35,** 748–751 (1975).
19. Limatibul, S., Shore, A., Dosch, H. M., Delfand, E. W.: Theophylline modulation of E-rosette formation an indicator of T-cell maturation. Clin. exp. Immunol. **33,** 503–513 (1978).
20. London, J., Berrih, S., Bach, J. F.: Peanut agglutinin. I. A new tool for studying T lymphocyte subpopulations. J. Immunol. **121,** 438–443 (1978).
21. Moretta, A., Mingari, M. C., Santoli, D., Perlman, P., Moretta, L.: Human T-lymphocyte alteration in systemic lupus erythematosus Scand. J. Immunol. **10,** 223–228 (1979).
22. Neubert, R., Sauer, H., Ambrosius, H., Leonhard. P.: Studies on the E rosette test. Allergie Immunol. (Leipzig) **26,** 361–367 (1980).
23. Palacios, R., Ruiz-Arguelles, A. Alarcon-Segovia, D.: Human post thymic precursor cells in health and disease. IX. Immunregulatory T cells in peripheral blood of patients with rheumatoid arthritis. Clin. exp. Immunol. **43,** 473–477 (1981 ).
24. Palacios, R., Alarcon-Segovia, D.: Human post thymic precursor cells in health and disease. Scand. J. Immunol. **13,** 499–502 (1981)
24. Palacios, R., Alarcon-Segovia, D., Llorente, L., Ruiz-Arguelles, A., Diaz-Jouanen, E.: Human post thymic precursor cells in health and disease. I. Characterization of the autologous rosette forming T cells as post thymic precursors. Immunology **42,** 127–135 (1981).
26. Palacios, R., Llorente, L., Ruiz-Arguelles, A., Alarcon-Segovia, D.: Human post thymic precursor cells in health and disease. V. Stimulatory effect of serum thymic factor on the cell cycle of autologous rosettes forming and T-cells. Immunol. Lett. **4,** 35–38 (1982).
27. Prithard, J. A., Seaman, J. E., Evans, I. H., James, K. W., Deeley, T. J., Kerby, I. J., Peterson, I. C. M.: Cancer specific density changes in lymphocytes after stimulation with phytohaemagglutinin. Lancet **1,** 1275–1277 (1978).
28. Rossen, R. D., Reisber, M. A., Hersch, G. M., Gutterman, J. U.: The $C_{1q}$ binding test for soluble immune complexes. Clinical correlations obtained in patients with cancer. J. nat. Cancer Inst. **58,** 1205–1216 (1977).
29. Rucheton, M., Caraux, J., Roy, A., Thiery, C., Esteve, C., Valles, H., Dufer, J.: Human autologous rosettes. II. Further characterization. Markers and functions. Cell. Immunol. **64,** 312–323 (1981).
30. Vose, B. M., Vanky, F., Fopp, M., Klein, E.: Restricted autologous lymphocytotoxicity in lung neoplasia. Brit. J. Cancer **38,** 375–381 (1978).
31. Yu, C. T.: Lymphocyte subpopulations. Human red blood cell rosettes. Clin. exp. Immunol. **20,** 311–322 (1975).

*Acta Medica Hungarica, 41 (2—3), pp. 129—134 (1984)*

*Nephrology*

# SHORT-TIME PERITONEAL DIALYSIS

I. Taraba, A. É. Balás, L. Fazakas, Andrea Hering, Katalin Török

NATIONAL INSTITUTE OF CLINICAL MEDICINE, BUDAPEST, HUNGARY

Received: November 27, 1982

On the evidence of observations comprising approximately 200 treatments by peritoneal dialysis (PD), the advantages of the weekly 3×9-h schedule over the 2×24-h schedule are pointed out. Utilization of dialysing fluid per week was 45.1 l versus 54.6 l. The improved general condition of the patients and the favourable changes of the biochemical parameters were in support of this dialysis strategy. The use of the type PDK-8 automated apparatus designed by the authors, is recommended as being simple to handle, labour-saving and apt to minimize the hazard of peritonitis.

**Keywords:** peritoneal dialysis, peritoneal clearance, dialysis treatment strategy, duration of dialysis

## Introduction

The number of patients requiring dialysis treatment for chronic renal failure has been sharply increasing in the last years, partly as a result of the improving diagnostic possibilities, and partly of the extension of the therapeutic services.

PD is no longer regarded as a last resort, but rather as an alternative to haemodialysis. The technique, indications, possibilities and limitations of the procedure are well documented [1, 5, 12, 15].

According to the data of E.D.T.A. the number of dialysis units with facilities for PD have been on the increase in Europe too. The figures from 1981 [8] show a fivefold increase in the number of PD patients over the last four years. On 31 December 1980 2749 patients in Europe were on PD. This implies that several hundreds of patients are receiving PD in various European countries, particularly in France, Italy, GFR and Great Britain.

The results obtained at this department have been reported earlier [14]. The present report deals with our strategy of peritoneal dialysis developed

Send offprint requests to I. Taraba, National Institute of Clinical Medicine, H-1088 Budapest, Szentkirályi u. 46, Hungary

*Abbreviations:* PD: peritoneal dialysis; HD: haemodialysis; CAPD: continuous ambulatory peritoneal dialysis; $C_p$: peritoneal clearance; $D_c$: concentration of given substance in the effluent; $SC_b$: serum concentration before dialysis; $SC_a$: serum concentration after dialysis; $V_d$: volume per min of dialysing fluid passing the abdominal cavity; $C_{ur}$: peritoneal urea clearance; $C_{creat}$: peritoneal creatinine clearance; $C_{ua}$: peritoneal uric acid clearance

along further lines providing for an increased performance of PD. It has been sought by this strategy to "reproduce", as it were, the function of the normal kidney by increasing the regularity of dialysis, while minimizing, as far as possible, the demands on the staff as well as on the patient and striving at the maximum possible efficiency. It was expected to achieve these objectives by a closer spacing of PD, by a reduction of the time of dialysis and by mechanization of the procedure.

## Patients and methods

Nine patients, five males and four females were studied. A total of 198 PD was performed. All patients had chronic renal failure when started on PD, the serum-creatinine levels being above 800 $\mu$mol/l and the daily urinary output below 500 ml in all of the cases.

The Tenckhoff-catheter serving for PD (Quinton QI 3244) was inserted under aseptic conditions in the surgical theatre from a low midline-incision.* In the early period PD was performed manually, later by means of the type PDK-8 automatic apparatus of our own design. This is a completely automated unit delivering 8 l dialysing fluid in the desired distribution (for details of the apparatus see our earlier report [10]). The present system allocates one hour for each cycle, 2 l dialysing fluid being instilled into and drained from the peritoneal cavity. Duration of inflow and outflow was 10 min for each, the time allowed for intraabdominal equilibration was 35 to 40 min. The 2 l volume was tolerated well and caused no discomfort to the patients. The volume of dialysing fluid utilized for "short-time" PD was thus 16 l, i.e. $2 \times 8$ l. The flasks with the fluid were kept in a prewarmer prior to PD and in a suspended warmer during instillation at a constant temperature of $37 \pm 1$ °C provided by electric heating.

The dialysing fluids were "Peridisol solutions "1D" and "2D", made by the HUMAN ("Institute for Vaccine Production and Research" Budapest) the composition of "Peridisol 1D" was, $Na+$: 140, $Ca^{++}$: 4, $Mg^{++}$: 1.5, $Cl^{-}$: 102, lactate 43.5 mval/l, glucose: 15 g/l. "Peridisol 2D" is of the same composition, with the difference that it contains 70 g/l glucose.

The serum creatinine, carbamide, uric acid, Na, K, Ca and P levels were measured before the start and after completion of PD. The serum total proteins, haemoglobin, haematocrit and leukocyte count were determined after PD. The patients were weighed before and after PD. Pulse-rate and blood pressure were checked during PD. Ten ml samples were taken from each successive litre of the outflowing dialysate and measured for the concentrations of creatinine, carbamide, uric acid, Na, K, Ca and P.

In accordance with published data [2, 4, 13], the peritoneal clearance of the given substance was calculated on the basis of the formula,

$$C_p = \frac{D_c \cdot V_d}{\frac{SC_b + SC_a}{2}}$$

where the numerator represents the quantity of the given solute cleared by PD per min, and the denominator the average serum concentration of the same solute.

$C_{perit}$ is thus equal to the quantity of the eliminated solute divided by the averages of its predialysis and postdialysis serum level.

As the first step of mathematical-statistical analysis of the data the histograms of the variables under study were plotted and the mean values and standard deviations were calculated. Furthermore, the total quantities of dialysed solutes per week and the products and quotients of the Ca and P levels were determined. The fluid input was correlated to the quantity of the dialysed substance by linear analysis of regression.

* We are indebted to Dr. L. Körmendi, member of the Research Staff of the National Institute of Traumatology, for his valuable assistance.

The data were checked, transformed and analysed partly on the basis of our own program, partly of the program package BMDP-77, using the computer R20 of the Section for Automated Analysis, Semmelweis University Medical School.

## Results

The results of a total of 198 short-time PD in nine patients, averaging 9 h in duration were analysed.

The predialysis and postdialysis serum levels of ions and small-molecular solutes have been summed up in Table I. The serum levels of the small-molecular substances retained in the serum have been found to decline as a result of dialysis. This was valid also for the serum K and P levels. On the other hand, there was a moderate increase in the Ca level.

**Table I**

*Serum levels of ions and small-molecular solutes (in S. I. units)*

| | n | Before PD mean ± S.E.M. | After PD mean ± S.E.M. |
|---|---|---|---|
| Creatinine | 197 | 935.29 ± 18.00 | 784.12 ± 15.39 |
| Urea | 196 | 31.47 ± 0.54 | 26.32 ± 00.54 |
| Uric acid | 198 | 679.85 ± 10.57 | 585.28 ± 10.82 |
| Na | 198 | 141.4 ± 0.19 | 141.9 ± 0.21 |
| K | 198 | 4.88 ± 0.058 | 4.16 ± 0.04 |
| Ca | 187 | 2.17 ± 0.01 | 2.19 ± 0.01 |
| P | 171 | 2.84 ± 0.04 | 2.36 ± 0.03 |
| Ca × P | 171 | 5.83 ± 0.08 | 5.11 ± 0.08 |
| Ca/P | 171 | 0.77 ± 0.02 | 1.003 ± 0.02 |

Average duration of PD: 9 h

The total dialysis time per week averaged 27 h, in other words, each dialysis lasted 9 h. The fluid volumes and the amounts of small-molecular substances cleared on a week are presented in Table II.

The utilized fluid volume (inflow) was correlated to the quantity of cleared small-molecular substances. The correlations between elimination and utilized fluid are expressed for carbamide and creatinine by the following equations of regression:

$$y = 0.0923\,x - 0.245$$

$$y = 1.059\,x + 3.93$$

**Table II**

*Weekly elimination by PD of small-molecular solutes* (n = 196)

| | Mean ± S.E.M. |
|---|---|
| Duration of dialysis | |
| min/week | 1623 ± 7.55 |
| h/week | 27 ± 0.11 |
| Water, ml | 4839 ± 263 |
| Urea, g | 28.07 ± 0.60 |
| Creatinine, g | 3.46 ± 0.09 |
| Uric acid, g | 3.02 ± 0.07 |
| Protein, g | 59.71 ± 2.72 |

Elimination of both substances from the organism increased in proportion to the volume of dialysing fluid. The volume utilised per treatment was 14 to 16 l (mean = 15 040 ml ± 268 ml). The fall in the serum levels and elimination of the given substance is shown in Tables and II.

From the changes in the serum levels and the elimination of a substance, its peritoneal clearance, as the indicator of the efficiency of dialysis, was calculated. By modifying the volume of utilized dialysing fluid and of the time of dialysis (equilibration time), the clearance values are also modified. The present "short-time" dialysis gives the following clearance values:

$C_{urea}$: 22.25 ± 5.71 ml/min (n: 190)

$C_{creat}$: 22.19 ± 5.41 ml/min (n: 193)

$C_{uric\ ac}$: 18.09 ± 5.83 ml/min (n: 193)

In two of the present cases we had changed over from the earlier long-time to short-time dialysis three times a week. The serum levels of the retained solutes are represented in Figs 1 and 2. In the interest of comparison, the serum carbamide, creatinine and uric acid levels during the last 10 weeks of long-time PD and those of the first 10 weeks of short-time PD are shown. The fall in the creatinine level was striking, that in the carbamide level was less so, and the uric acid level showed a minor increase. PD three times a week caused less discomfort to the patients and was far less exhausting than the two admissions of the weekly 24 h of dialysis. Short-time dialysis on an out-patient basis was tolerated much better by patients, and the satisfactory control of blood chemistry was accompanied by an improved sense of well-being.

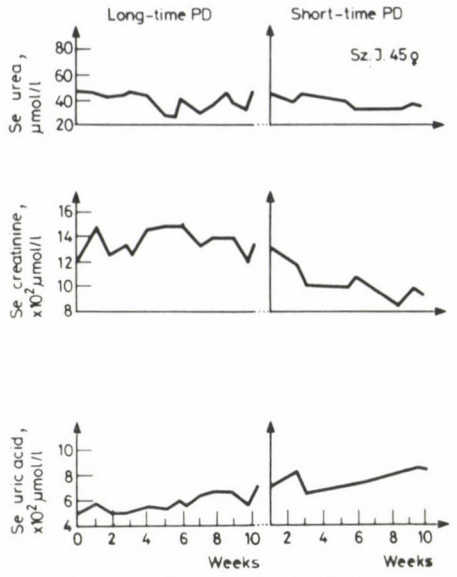



*Fig. 1.* The serum levels of the retained solutes (short-time dialysis)

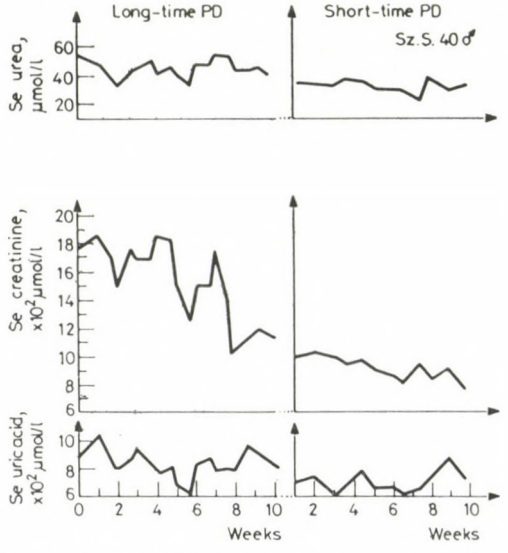



*Fig. 2.* The serum levels of the retained solutes (short-time dialysis)

## Discussion

In the cases reported earlier chronic PD had been applied to patients twice a week for 24 h. This form of treatment was exhaustive to the patients, even in the absence of any complication. Therefore, many authors have

attempted to shorten the period of dialysis [3, 6, 7, 9, 11, 13, 16], obviously without reducing its efficacy.

While the function of the normal kidney is continuous, dialysis treatment occurs periodically. The ideal objective would be, therefore, a continuous dialysis reproducing the function of the normal kidney. Continuous dialysis involving hospitalization is, however, beyond endurance. On the other hand, continuous ambulatory peritoneal dialysis (CAPD) introduced recently makes it possible to solve it by home-dialysis. Closely spaced short-time PD, though providing no CAPD, approximates the physiological kidney function more than does long-time PD carried out in long intervals.

The patients forming the present material received three treatments per week, routinely applied in HD as well. By this practice the withdrawal of retained fluid was smooth and elimination of the retained substances more continuous.

Comparison of the efficacy of 9-h and 24-h treatments showed a slighter fall in the serum levels after the 9-h treatments. By increasing the volume of dialysing fluid to 2 l per cycle we were able to increase the efficiency of dialysis per unit of time, as reflected in the values of peritoneal clearance. A closer spacing of the treatments allowing to approximate the physiological function of the kidney as far as possible. Comparison of the total fluid volumes utilized per week, of the duration of dialysis in hours and of the levels of retained substances in the serum clearly showed that utilization of 45 l fluid per week within 27 h per week produced a more marked fall, or a less marked increase, in the levels of the retained substances than had a PD of 46 h with 54.6 l fluid weekly. The short-time PD was thus superior in effect to the long-time treatment (Table III).

If the results of long-time and short-time PD are represented graphically on the assumption that the serum level of a solute is at its mean value before dialysis, then a saw-tooth shaped graph is obtained (Fig. 3). In the case of

**Table III**

*Comparison of short-time and long-time PD*

| | | Short-time PD 3 treatments weekly mean ± S.D. | Long-time PD 2 treatments weekly mean ± S.D. |
|---|---|---|---|
| Time of dialysis per week | h | 27.0 | 46.5 |
| Amount of dialysing fluid per week | liter | 45.1 ± 7.0 | 54.6 ± 5.3 |
| Serum levels before dialysis | Urea, mmol/l | 31.4 ± 7.38 | 41.39 ± 9.01 |
| | creatinine, μmol/l | 935.2 ± 250.1 | 1309.2 ± 287.3 |
| | uric acid, μmol/l | 679.8 ± 146.9 | 731.6 ± 133.2 |

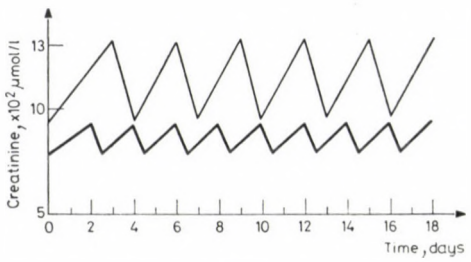



*Fig. 3.* The serum level of a solute after long-time and short-time PD

a closer spacing of the treatments the rise in the serum-levels is less sharp and its fall during dialysis is smoother than in the case of longer intervals. (The CAPD referred earlier is theoretically still more physiological than short-time PD, by providing for a more continuous excretion.)

A substantial proportion of the treatments under review were carried out by means of an automatic or semiautomatic apparatus [10]. This provides for the delivery of $2 \times 8$ l fluid in 8 to 9 hours, inflow and outflow being controlled on the basis of a predetermined programme. It is reliable and permits reading of the fluid balance at any desired moment, thus providing for the continuity of monitoring. Electric heating helps to minimize the hazard of peritonitis involved by heating on a water-bath.

On the evidence of close to 200 PDs performed in nine patients, short-time-PD by the use of an automatic apparatus was found effective and less exhausting than long-time-PD. The patients have to return to hospital more often, but the stay in hospital is shorter, therefore the conditions of rehabilitation are better than in case of long-time HD. By dispensing with 24-hour dialysis extending over the nocturnal hours, the procedure makes less demands on manpower. The shorter duration of dialysis is beneficial to the general condition, despite the reduction in the total volume of dialysing fluid, reflected also in the laboratory findings. Two of the present cases in which short-time dialysis has been substituted for earlier long-time PD, are particularly illustrative of the advantages of the former.

## REFERENCES

1. Blumenkrantz, M. J., Lindsay, R. M.: Comparisons of hemodialysis and peritoneal dialysis. Contributions to Nephrology **17,** Vol. S. Karger, Basel 1979, pp. 20–29.
2. Brown, E. A., Kliger, A. S., Finkelstein, F. O.: Peritoneal dialysis clearance. Nephron **21,** 310–316 (1978).
3. Cambi, V., Savazzi, G., Arisi, L., Bignardi, L., Bruschi, G., Rossi, E., Migone, L.: Short dialysis schedules finally ready to become a routine? Proc. EDTA **11,** 112–118 (1974).
4. Finkelstein, F. O., Kliger, A. S., Bastl, C., Pepita, Y.: Sequential clearance and dialysance measurements in chronic peritoneal dialysis patients. Nephron **18,** 342–347 (1977).
5. Gahl, G. M., Kessel, M.: The indwelling stylet catheter for peritoneal dialysis. Clin. Nephrol. **6,** 414–415 (1976).

6. Giordano, C., De Santo, N. G., Papa, A., Capodicasa, G., Cirillo, D., Capasso, G., Quarto, E., Gallo, B., Sanna, G.: Short daily peritoneal dialysis. Kidney Int. **7,** 425–430 (1975).
7. Giordano, C., De Santo, N. G., Cirillo, D., Capodicasa, G., Rinaldi, S., Cicchetti, T., Di-Maio, F.: Short daily peritoneal dialysis and protein restriction. Proc. EDTA **12,** 132–138 (1975).
8. Jacobs, C., Broyer, M., Brunner, F. P., Brynger, H., Donckerwolcke, R. A., Kramer, P., Selwood, N. H., Wing, A. J., Blake, P. H.: Combined report on regular dialysis and transplantation in Europe. Proc. EDTA **18,** 4–58 (1981).
9. Kjellstrand, C. M., Evans, R. L.: Considerations of new dialysis schedules: Theoretical evaluation and review of literature. Opusc. med. techn. (Lund) **16,** 26–37 (1975).
10. Kottra, G., Taraba, I., Wahl, F.: Automata peritonealis dializis készülék (An automatic apparatus for peritoneal dialysis) Orvos Techn. **17,** 172–177 (1979).
11. Manji, T., Maeda, K., Ohta, K., Saito, A., Amano, A., Kawaguchi, S., Kobayashi, K.: Short Time Dialysis. Proc. EDTA **12,** 589–595 (1975).
12. Oreopoulus, D. G.: Chronic peritoneal dialysis. Clin. Neprhol. **9,** 165–173 (1978).
13. Paola, M., Messina, M., Segoloni, G. P., Nebiolo, P. E., Veronesi, G. V., Quarello, F., Piccoli, G., Vercellone, A.: Short dialysis strategy: six years' experience. Dial. Transpl. **11,** 220–222 (1982).
14. Taraba, I., Spustova, V., Balás, É. A., Dzurik, R., Petrányi, Gy.: Peritoneal dialysability of "Uraemic Substances". Int. Urol. Nephrol. **13,** 193–198 (1981).
15. Tenckhoff, H.: Peritoneal Dialysis Today: A New Look. Nephron **12,** 420–436 (1974).
16. Vercellone, A., Piccoli, G., Alloatti, S., Segoloni, G., Stratta, P., Giachino, G., Coppo, R., Buffa, C.: Short dialysis: indications and limits. Opusc. med. techn. (Lund) **16,** 12–24 (1975).

*Acta Medica Hungarica, 41 (2—3), pp. 137—141 (1984)*

# EFFECT OF LEVAMISOLE ON CELL SENSITIVITY IN RATS WITH EXPERIMENTAL HEYMANN'S NEPHRITIS

I. E. Tareyeva, S. N. Savitsky, N. B. Gordovskaya, V. A. Varshavsky

LABORATORY OF NEPHROLOGY, 1ST MOSCOW MEDICAL SETCHENOV INSTITUTE
MOSCOW, USSR

Received: March 10, 1983

The development of cell-sensitization to renal antigen in rats with experimental Heymann's glomerulonephritis and the effect of levamisole on cell sensitivity have been studied. Morphologic changes in GN rats appeared after 14 days and were most pronounced on days 21–28, with focal proliferation of glomerular cells, an increase of mesangium, a focal increase of the basal membrane material. After day 49 the changes improved slightly and were still the same on day 147 when a location of IgG to the glomerular basal membrane was detected. In every GN rat, cell-sensitivity to renal antigen was revealed from day 21 of the experiment till its end. Levamisole treatment decreased renal morphologic changes and abolished cell-sensitivity to renal antigen. The possible mechanisms of preventing the development of cell-sensitization in rats treated with levamisole are discussed.

**Keywords:** experimental glomerulonephritis, cell-sensitization, levamisole

## Introduction

The immunostimulator levamisole is widely used in the treatment of rheumatological, oncologic, and infectious diseases accompanied by a decreased cell-mediated immunity (CMI). It has been shown that levamisole helps to restore the functions of polynuclear leucocytes, macrophages [3, 18, 19] and T-cells [4, 5]. In glomerulonephritis (GN), disfunction of the CMI takes place, which is manifested in the acute period by a reduction of the total number of T-lymphocytes and by a decrease in their functional activity [11, 17], suppression of the phagocytic function [7] as well as by the appearance of lymphocytes sensitized to renal antigens [9, 14]. These facts have justified an attempt to use levamisole in the treatment of GN.

There are few studies discussing the effect of levamisole on renal processes, and most of the few available refer to the treatment of lupus nephritis in humans and NZB/W mice.

The purpose of this work was to study the effect of levamisole on the course of experimental nephritis and on the dynamics of cell-sensitization to the specific renal antigen in the development of experimental nephritis.

Send offprint requests to I. E. Tareyeva, Laboratory of Nephrology, 1st Moscow Medical Setchenov Institute 11a Rossolimo st. 119021 Moscow, USSR

## Materials and methods

The study was performed on 120 female Wistar rats weighing 200 to 250 g (Laboratory Animals Institute, USSR Academy of Medical Sciences). Before the experiment, all animals were quarantined for one month.

Renal antigen (RA) was obtained from kidneys of 55 rats of a homologous line. Protein was determined according to Lowry et al. Migration inhibition test (MIT) was performed by the direct capillary method of Søborg and Bendixen [15] with modifications suggested by Mazharov et al. The concentrations of RA used in the experiment, 50, 100 and 300 mcg/ml, did not cause non-specific migration of donor cells. The MIT was considered positive if the migration index was below 0.8.

Experimental glomerulonephritis (EGN) was created according to Heymann et al. [8] by intraperitoneal injections of RA (50 mg per kg) in complete Freund adjuvant (Difco Laboratories, Detroit; 0.5 ml per injection) weekly for 6 weeks. The schedule of the experiment is shown in Table I.

**Table I**

*Schedule of the experiment*

| Groups of rats | Renal antigen | Complete Freund adjuvant | Levamisole |
|---|---|---|---|
| group 1 (n : 20) | + | + | — |
| group 2 (n : 20) | + | + | + |
| group 3 (n : 5) | — | — | — |
| group 4 (n : 5) | — | — | + |
| group 5 (n : 5) | — | + | — |

n = number of rats

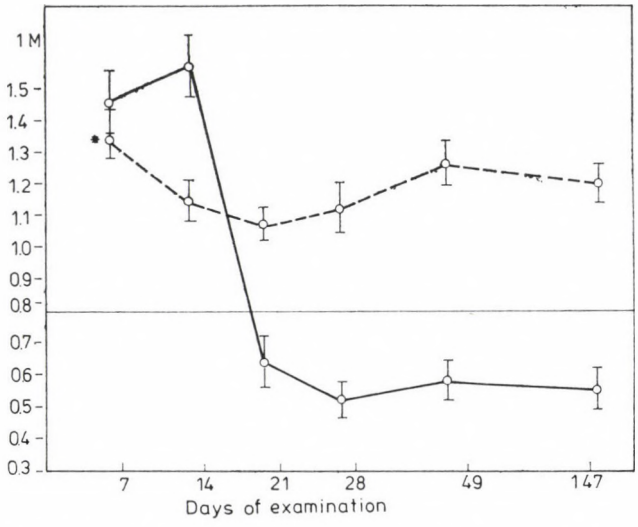



*Fig. 1.* MIT with renal antigen in experimental glomerulonephritis (mean ± S.E.M.)

Group 1: rats receiving injections of RA 50 mg/kg + 0.5 ml of 0.9% physiological salt solution +0.5 ml of Freund's complete adjuvant.

Group 2: rats receiving injections of RA 50 mg/kg + 0.5 ml of 0.9% physiologic salt solution +0.5 ml of Freund's complete adjuvant +4.5 mg/kg of levamisole.

Group 3: intact rats.

Group 4: rats receiving 4.5 mg/kg of levamisole.

Group 5: rats receiving 0.5 ml of 0.9% physiological salt solution +0.5 ml Freund's complete adjuvant.

The dosage of levamisole (Decaris, G. Richter, Budapest) was calculated on the basis of the average human dose [6].

Animals belonging to groups 2 and 4 received hypodermic injections of levamisole 3 times a week from the beginning of the experiment for 65 days.

The animals were killed under ether anaesthesia at 7, 14, 21, 28, 49 and 147 days by exsanguination. The kidneys of three animals for each day were used for morphological examination. They were fixed immediately in formalin, and stained with haematoxylin-eosin, van Gieson, Asan and PAS. For immunofluorescent study the direct method of Coomb's was used.

## Results

In group 1 after day 14 there was a slight proliferation of glomerular cells, a focal broadening of mesangium, a mild thickening of the basal membranes of capillary loops. On days 21–28 glomerular cell proliferation and expansion of the mesangium became more marked and doubling of basal membranes and sclerosis of some glomerular loops appeared. On day 49 the histologic changes decreased slightly and were the same on day 147. Tubular and interstitial changes were similar throughout the study: the tubular epithelium was in the state of granular degeneration; hyperaemia of vessels and mild focal lympho-histiocytic infiltration were noted.

Immunofluorescent examination was negative on all days except the 147th. On the 147th day a fixation of rat gamma-globulin on the renal basement membranes was noted, the fluorescence being of focal granular character.

In the second group the changes were less significant: mild proliferation of glomerular cells, mild focal thickening of glomerular basement membranes, granular degeneration of tubular epithelium, rare interstitial mononuclear infiltrates were observed. Immunofluorescent study was negative on all days.

In the three control groups, there were no histologic changes in the kidneys and immunofluorescent studies were negative.

When the first group was studied for cell sensitization, an increase in migration indices was marked on the 7th and 14th day of experiment. The average indices were 1.45 and 1.56 respectively. From the beginning of the 21st day, a distinct inhibition of migration was marked in this group. The indices remained low to the end of the experiment (0.65, 0.52, 0.57 and 0.54, respectively).

In the second group an increase in migration indices was marked (average, 1.34), just as in the first group. Later, however, in contrast to the first group, the migration index remained normal throughout in each of the groups, showing no difference from those of the control group (average indices were 1.15, 1.07, 1.10, 1.27 and 1.17, respectively).

## Discussion

In our study the migration inhibition test demonstrated the development of cell hypersensitivity to renal antigen in rats with experimental Heymann's nephritis and the influence of levamisole on cell sensitivity.

In patients with chronic GN cell sensitivity to renal antigens occurs in 17–80% of the cases, more frequently in the active periods of the disease [9, 12, 14]. In our previous studies cell-sensitivity to GBM antigen was demonstrated in 81.3% of chronic GN patients [13].

Few studies have dealt with cell-sensitivity to renal antigens in experimental animals. Litwin et al. [10] found positive skin tests with renal tubular antigen ($F_x1A$) being responsible for the glomerular injury in rats with Heymann's nephritis. The positive skin tests correlated with the blast transformation of lymphocytes. Bakker et al. [2] demonstrated MIF production to specific renal antigen in the same experimental model. The role of sensitized cells in the genesis of glomerulonephritis is unclear, and no serial investigations into cell-sensitization during GN development have been reported.

In our studies of rats with Heymann's nephritis morphological changes in the kidneys appeared after day 14 and were the most significant on days 21–28. The changes observed were similar to those reported by Heymann et al. [8], Avasthi et al. [1] and Bakker et al. [2]. Immunofluorescent examination was always negative except on the 147th day, when a fixation of rat gammaglobulin to the renal basement membranes was noted, the fluorescence being of focal granular character. All GN rats showed cell-sensitivity to renal antigen from day 21 of the experiment till the end.

Thus, sensibilization was expressed earlier than the deposition of immune complexes could be revealed, histological changes had preceded the development of cell-sensitization.

Our results showed that changes in cell-mediated immunity were not responsible for initiating the disease, but they might be responsible for its maintenance.

The second question was the influence of levamisole on the development of cell sensitivity. Morphological changes were less pronounced in kidneys of rats treated with levamisole and immunofluorescent studies were negative. Cell sensitivity to renal antigen was not observed in any of these animals.

Thus, levamisole did not prevent the development of experimental glomerulonephritis, though somewhat decreased the morphological changes. At the same time, it prevented the development of cell sensitivity to renal antigen, a fact, that seems to be of considerable interest.

The mechanism by which levamisole prevents the development of cell sensitization is not clear. The disappearance of the migration inhibition factor

may be connected with the restoration of the immunoregulating function of T-cells, and an immediate blocking effect of levamisole on the given population cannot be excluded, either [16].

The diminution of morphological changes and the absence of cell sensitivity in the animals treated with levamisole permits to suggest the use of immunostimulating therapy in the early stages of Bright's disease.

## REFERENCES


1. Avasthi P. S., Avasthi P., Tokuda, S., Anderson R. E., Williams R. S.: Experimental Glomerulonephritis in the mouse. Clin. exp. Immunol. **9,** 667–676 (1971).
2. Bakker, W. W., Mulder I., Lee, R., Fleuren G. J., Hoedemaeker Ph. J.: Experimental Glomerulonephritis in the rat induced by antibodies directed against tubular antigens. Int. Arch. Allergy **54,** 405–413 (1977).
3. Chumakova M. M.: Some aspects of levamisole action. Ter. Arkh. **9,** 146–153 (1978)
4. Goebel K., M. Schubota K., Goebel F. D.: Levamisole-induced immunostimulation in Spondylarthropathies. Lancet, **2,** 214–217 (1977).
5. Gonzales M., Rodriguer-Escobedo L., Castanedo de Alba G. P.: Effect of levamisole on E-rosettes. New Engl. J. Med. **295,** 230–236 (1976).
6. Gordon B. L., Yanagihara R.: Treatment of systemic lupus erythematosus with the T-cell immunopotentiator levamisole: A follow-up report of 16 patients under treatment for a minimum period of four months. Ann. Allergy **39,** 227–236 (1977).
7. Gordovskaya N. B., Shilov E. M., Savitsky S. N., Miroshnichenko N. G., Mukhin N. A., Tareyeva I. E.: Effect of levamisole on the immunological indices in patients with chronic glomerulonephritis. Ter. Arkh. **4,** 28–31 (1980).
8. Heymann, W., Hackel D. B., Harwood S., Wilson S. G. F., Hunter J. L. P.: Production of nephrotic syndrome in rats by Freunds adjuvants and rat kidney suspensions. Proc. Soc. exp. Biol. (N.Y.) **100,** 660–664 (1959).
9. Janas-Boratynska M.: Cell-mediated hypersensitivity in glomerulonephritis. Arch. Immunol. **27,** 15–27 (1979).
10. Litwin A., Adams L. E., Levy R., Cline S., Hess E. V.: Cellular immunity in experimental glomerulonephritis of rats. Immunology **20,** 755–766 (1971).
11. Lenchic V. I., Poljantseva L. R., Sura V. V., Tareyeva I. E. Lymphocyte blasttransformation test in chronic kidney diseases. Ter. Arkh. **4,** 60–63 (1975).
12. Matsumoto K., Yoshizawa N., Hatano M.: Studies of cell-mediated immunity in human glomerulonephritis by macrophage migration inhibition test. Nephron **21,** 192–200 (1978).
13. Savitsky S. N., Gordovskaya N. B., Shilov E. M., Trayanova T. G.: Specific sensitization of lymphocytes in some nephropathies. Urol. Nephrol. **3,** 18–23 (1980).
14. Schmitt E., Seyfarth M., Werner H., Klinkmann H.: Cellular immunity in glomerulonephritis. Clin. Nephrol. **17,** 271–276 (1982).
15. Søborg M., Bendixen G.: Human lymphocyte migration as a parameter of hypersensitivity. Acta med. Scand. **181,** 247–256 (1967).
16. Symoens G., Rosenthal M.: Levamisole in the modulation of the immune response: the current experimental and clinical state. A review. J. Reticuloend. Soc. **21,** 175–221 (1977).
17. Tareyeva I. E., Shilov E. M., Gordovskaya N. B.: The effects of azathioprine and prednisolone on T and B-lymphocytes in patients with lupus nephritis and chronic glomerulonephritis. Clin. Nephrol. **14,** 233–237 (1980).
18. Van Heule R., De Gree G., Adriaenssens K., De Hauwere R.: Levamisole therapy for cellular immunologic deficiency with high IgE values. Acta paediat. Belg. **29,** 41–43 (1976).
19. Verhaegen H., De Cock W., De Gree G., Verbruggen F., Verhaegen-Declerco M.: In vitro restoration by levamisole of thymus-derived lymphocytes function in Hodgkin's disease. Lancet, **1,** 978–979 (1975).

*Acta Medica Hungarica, 41 (2—3), pp. 143—147 (1984)*

## *Gastroenterology*

# BILE ENZYME ACTIVITIES FOLLOWING CHOLEDOCHOTOMY AND THE EFFECT OF STEROID TREATMENT

I. Édes[1], I. Édes[2] jr.

[1]KISKUNFÉLEGYHÁZA HOSPITAL, [1]DEPARTMENT OF SURGERY KISKUNFÉLEGYHÁZA, AND [2]SECOND DEPARTMENT OF MEDICINE, UNIVERSITY MEDICAL SCHOOL, SZEGED, [1]HUNGARY

Received: December 28, 1982

The alkaline phosphatase (AP) and gamma-glutamyl-transpeptidase (GGT) activities and the effect of steroid treatment were studied in human bile and serum following choledochotomy. Activity of the two enzymes in the bile changed in parallel. Following the operation the enzyme activities initially decreased, but after 3 days they showed a progressive increase and reached their maximum on day 6. It appears that the bile is a major route for the elimination of both enzymes from the damaged liver.

60 mg Prednisolone on the day of operation and 2 days after surgical intervention prevented the initial decline in enzyme activity and significantly increased the excretion of enzymes. The possible pathogenesis of enzymatic changes after choledochotomy is discussed.

**Keywords:** choledochotomy, bile enzymes, steroid treatment

## Introduction

Cholestatic disorders are usually associated with increased serum activities of gamma-glutamyl-transpeptidase (GGT, EC 2.3.2.2.) and alkaline phosphatase (AP, EC 3.1.3.1.) [2, 11]. Delayed clearance from the circulation and enzyme leakage due to the hepatic injury caused by bile duct obstruction are responsible for the prolonged elevation of the enzyme activities. Alternatively, since both GGT and AP are inducible enzymes [9], high serum levels may occur because the hepatic synthesis exceeds the rate of enzyme elimination. Biliary obstruction is known to stimulate the hepatic synthesis of AP, with a resulting elevated serum activity of the enzyme [6, 7].

Fewer data are available in the literature as to the enzyme activities in human bile. High levels of GGT and AP were found in the bile of patients with T-tube drainage following cholecystectomy [1], but the pathogenesis of enzymological changes following surgical correction has not been clearly

Send offprint requests to I. Édes, Kiskunfélegyháza Hospital, H-6100 Kiskunfélegyháza, Fadrusz u. 4., Hungary

established. We have therefore studied the mechanism of biliary enzyme excretion in T-tube bile collected during 14-day postoperative period in patients undergoing choledochotomy, in an attempt to acquire a more complete understanding of the enzymological changes after surgery.

## Patients and methods

The study was performed in 21 patients, 18 females and 3 males, with T-tube drainage following cholecystectomy and exploration of the common duct, performed for the removal of stones from the gall bladder and bile ducts. In all cases the postoperative diagnosis was choledocholithiasis with cholecystolithiasis.

Fourteen patients were slightly jaundiced before operation, with serum bilirubin levels less than 50 $\mu$mol/l. Patients with higher serum bilirubin levels than 50 $\mu$mol/l were excluded. Prophylactically, to all patients tetracycline or in some cases ampicillin plus oxacillin were administered intravenously. No postoperative complication was observed. Bile samples were taken from the common duct at operation and from the T-tube during 14-day postoperative period. At the same time serum samples were also collected for enzymatic determinations.

In 6 patients, 60 mg/day 21-deoxy-21-N-/N'-methyl-piperazinyl/-prednisolone(Depersolon, G. Richter, Budapest, Hungary) was adminstered intravenously on the day of operation and on the first and second postoperative days. These patients were slightly jaundiced and there was no contraindication to steroid treatment.

The bile samples were centrifuged at 3000 *g*, at 4 °C for 30 min and the clear supernatants were used for analysis. AP and GGT activities were measured by means of Boehringer test Kits. To avoid the effects of dilution and concentration on the results, the enzyme activities in the bile were expressed as mU/mg protein. Serum activities are given as mU/ml. Protein content was determined according to Peterson [8], with bovine serum albumin as the tsandard.

Statistical evaluation of the results was done by Student's *t* test.Values with $P < 0.05$. were regarded as significant statistically.

## Results

Characteristic parallel changes were observed in the activities of GGT and AP in the bile samples following the surgical intervention. The activities of both enzymes initially decreased from the values in the samples obtained during operation (Fig. 1). Three days after operation the levels of GGT and

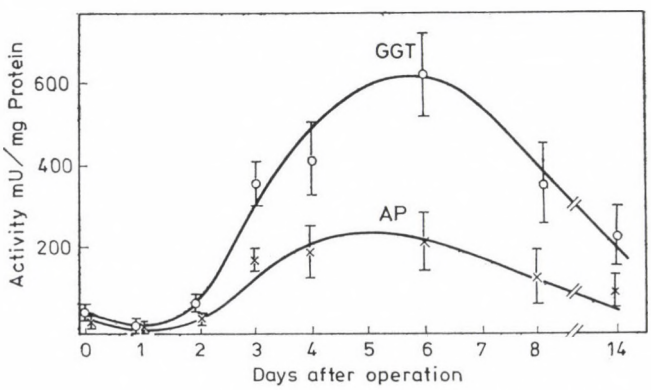



*Fig. 1.* AP and GGT activities in bile following choledochotomy
Day 0 relates to samples taken during operation

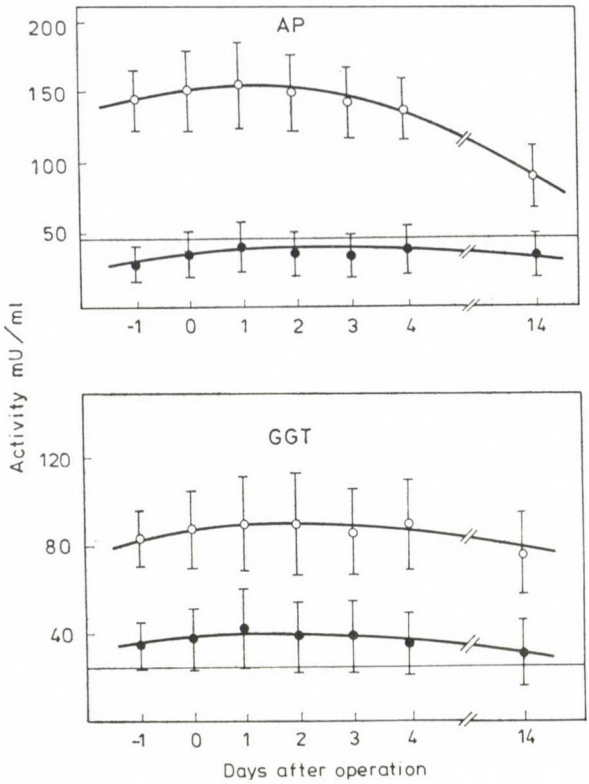



*Fig. 2.* AP and GGT activities in serum following choledochotomy. Day -1 relates to samples taken one day before the operation. Day 0 refers to samples taken 2–3 hours after operation. The solid line (—) shows the upper normal limit of enzyme activities. The open circles (○—○) represent jaundiced patients, and the filled circles (●—●) the group with normal serum bilirubin levels

AP were markedly elevated in the bile, reaching a peak on the 6th postoperative day. Thereafter a slight gradual decline was observed but 2 weeks after operation the activities were still high. It did not seem to make any difference whether the patients had been jaundiced or not.

Figure 2 shows the serum AP and GGT activities. There was a pronounced difference between jaundiced patients and those who had no jaundice. In the case of jaundiced patients, the AP showed a high activity and a gradual decline after the operation, while in those without jaundice it remained mainly under the upper normal limit during the test period. In serum GGT activity surgery did not cause significant changes the values were over the upper normal limit in every patient.

In six patients treated with prednisolone the steroid did not cause significant changes in the serum AP and GGT activities as compared to the

**Table I**

*Enzymes' activity in bile of patients treated with steroid*

| Days after operation | Without steroid treatment | | Steroid-treated | |
|---|---|---|---|---|
| | AP | GGT | AP | GGT |
| 0 | 19.9 ± 5.8° | 40.2 ± 8.3 | 20.1 ± 6.6 | 45.0 ± 9.2 |
| 1 | 6.2 ± 2.8 | 12.2 ± 3.4 | 18.9 ± 5.8* | 43.2 ± 12.4* |
| 2 | 32.3 ± 8.8 | 56.8 ± 8.3 | 69.9 ± 10.3* | 180.0 ± 35.6* |
| 3 | 172.0 ± 28.8 | 355.1 ± 49.2 | 188.3 ± 30.3 | 363.7 ± 52.3 |

° mean ± S.D.
* significantly different from the value observed in the group without steroid treatment. Day 0 refers to samples taken during operation.

patients who had no steroid treatment. The effect of this treatment on the enzymes in the bile is shown in Table I. The initial decline was prevented and two days after the operation significantly higher AP and GGT activities were observed in the bile samples. In the late postoperative period the enzyme activities of patients with and without steroid treatment did not differ significantly.

## Discussion

The high enzyme activities in human bile following choledochotomy may be regarded as a washout effect. Enzymes in the bile presumably originate largely from the liver, or at least from the biliary system, since a significant hepatic clearance and excretion of such large molecules from the serum is unlikely [6]. This was confirmed by gel electrophoretic examination of the AP isoenzymes in bile, since only the liver AP isoenzyme variant has been detected [4].

Kaplan and Righetti [7] observed a marked hepatic induction of AP in response to bile duct ligation in the rat. Owing to the obstruction, the increased enzyme contents in the liver are regurgitated into the serum. This is the most likely explanation for the high enzyme activities found in cholestatic disorders in humans [1]. The precise stimulus of the AP increase in cholestasis remains obscure. In cholestasis of any cause the failure of bile flow exposes the liver to increased concentrations of compounds which normally are excreted with the bile. Potentially the most active compounds are the bile acids, which have been shown to stimulate the increase of AP activity in rat liver cell cultures [5]. Following surgical correction, after a short initial period a pronounced biliary enzyme excretion was observed. The increased elimination of enzymes and the cessation of the increased enzyme induction

are responsible for the rapid normalization of AP activity in the serum of jaundiced patients. The serum GGT activity remained however high for several weeks after the surgical relief of obstruction and at a time when the serum bilirubin values have decreased to normal [3]. Presumably, since GGT is a sensitive indicator of liver lesion, its rate of elimination with the bile is insufficient to remove all the enzymes released by the damaged liver, and regurgitation continues for some time after the surgical correction.

Following choledochotomy, decreases were initially observed in the AP and GGT activities of human bile, and a gradual increase in the enzyme contents occurred only two days after the operation. The initial decline was presumably caused by a slight, temporary postoperative cholestasis. This was supported by the result of steroid treatment, which prevented the decrease in activity and significantly increased the excretion of enzymes. This slight postoperative damage in liver function could not be detected in serum, since we did not observe significant changes in the serum GGT and AP activities. Glucocorticoids, however, may alter the rate of synthesis of certain forms of ribonucleic acid and induce in tissues the synthesis of several enzymes including AP [6, 10]. Alternatively, the induction of enzymes may also contribute to the increased bile activities during steroid treatment.

**Acknowledgement**

We are indebted to Dr. F. Bodor, Dr. Gabriella Horváth, Miss Hermina Bajnóczi and Mrs Mária Bense for excellent technical assistance.

REFERENCES

1. Baker, P. R., Cushieri, A.: Bile enzyme activities in patients with T-tube drainage following cholecystectomy. Brit. J. exp. Path. **59,** 277–281 (1978).
2. Combes, B., Schenker, S.: Laboratory test. In: Schiff, L. (ed.): Diseases of the liver. 4th ed, Lippincott, Philadelphia 1975.
3. Cushieri, A., Baker, P. R.: Gamma-glutamyl-transpeptidase in hepato-biliary disease, value as an enzymatic liver function. Brit. J. exp. Path. **55,** 110–115 (1974).
4. Édes, I., Édes I. jr.: A humán epe enzimtartalmának változása choledochotomiát követően. Magy. Seb. **35,** 343–346 (1982).
5. Hatoff, D. E., Hardison, W. G. M.: Induced synthesis of alkaline phosphatase by bile acids in rat liver cell culture. Gastroenterology **77,** 1062–1067 (1979).
6. Kaplan, M.: Alkaline phosphatase. Gastroenterology **62,** 452–468 (1972).
7. Kaplan, M., Righetti, A.: Induction of rat liver alkaline phosphatase. The mechanism of the serum elevation in bile duct obstruction. J. clin. Invest. **49,** 508–516 (1970).
8. Peterson, G. L.: A simplication of the protein assay method of Lovry et al. which is more generally applicable. Anal. Biochem. **83,** 346–356 (1977).
9. Rosalki, S. B., Tailow, D., Rau, D.: Plasma gamma-glutamyl-transpeptidase elevation in patients receiving enzyme-inducing drugs. Lancet **2,** 376–377 (1971).
10. Thompson, E. B., Lippman, M. E.: Mechanism of action of glucocorticoids. Metabolism **23,** 159–202 (1974).
11. Varga, L., Sohár, I., Döbrönte, Z.: A gamma-glutamyl-transpeptidase enzim szintje máj és epeút betegségekben. Orv. Hetil. **117,** 201–203 (1976).

*Acta Medica Hungarica, 41 (2—3), pp. 149—155 (1984)*

# EFFECT OF DIFFERENT PROSTAGLANDIN ANALOGUES ON GASTRIC ACID SECRETION AND MUCOSAL BLOOD FLOW IN THE DOG: ACTION ON STIMULATED AND RESTING MUCOSA

G. A. BÁLINT, I. SÁGI[1], Z. DÖBRÖNTE, V. VARRÓ

FIRST DEPARTMENT OF MEDICINE AND [1]INTENSIVE CARE UNIT, UNIVERSITY MEDICAL SCHOOL, SZEGED HUNGARY

Received: April 20, 1983

The effect of PG-$E_2$, 16-16-DMPG$E_2$, PG-$F_{2\alpha}$ and PG-$I_2$ was investigated on histamine stimulated and resting canine gastric mucosa. PG-$F_{2\alpha}$ had no effect on mucosal circulation or acid secretion. PG-$E_2$ and 16-16-DMPG$E_2$ showed an increasing effect on blood flow of the resting mucosa. PG-$E_2$, 16-16-DMPG$E_2$ and PG-$I_2$ decreased of acid secretion; the effect seemed to be due to a direct action on the parietal cells.

**Keywords:** prostaglandins, stomach, acid secretion, mucosal blood flow

## Introduction

PGs are widely distributed in various tissues including the gastric mucosa. Several studies have demonstrated that the gastric mucosa is capable of synthesizing various PGs, which have been suggested to exert local, negative feed-back inhibition of gastric secretion and to act as modulators of gastric circulation [1, 8].

The present study was designed to compare in dogs the effect of different PG-analogues administered directly into an artery supplying the stomach, on acid secretion and gastric MBF after histamine stimulation or at rest of gastric mucosa.

## Materials and methods

Fourty seven mongrel dogs of both sexes, weighing 12–18 kg, were deprived of food for 48 h (water ad libitum), before experimentation. The animals were anaesthetized with pentobarbital (30 mg/kg, i.v.). The operative procedure has been described earlier [10].

Gastric MBF was determined by the aminopyrine clearance technique [3, 5]. The clearance of aminopyrine as an estimate of MBF was expressed in ml/min. A ratio (R) was calculated reflecting the relationship between the gastric MBF and the rate of gastric secretion.

Send offprint requests to G. A. Bálint, 1st Dept. of Medicine, Univ. Med. School, H-6701 Szeged, P.O. Box 469, Hungary

Abbreviations: PG: prostaglandin; MBF: mucosal blood flow; PG-$I_2$: prostacyclin; 16-16-DMPG$E_2$: 16-16-dimethyl-PG-$E_2$

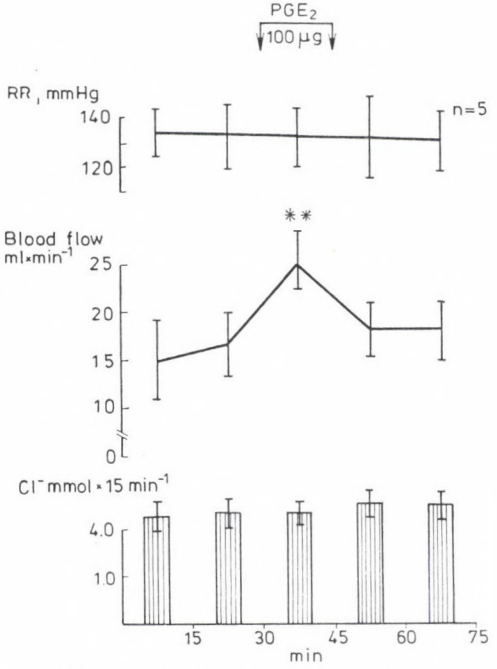



*Fig. 1.* Effect of PG-$E_2$ on resting gastric mucosa. n = number of dogs. Top: systemic blood pressure. Middle: aminopyrine clearance (MBF). Bottom: chloride secretion in 15 min periods, mean ± S.D.** = $P < 0.01$ vs 1st period. Arrows indicate the duration of PG-$E_2$ infusion

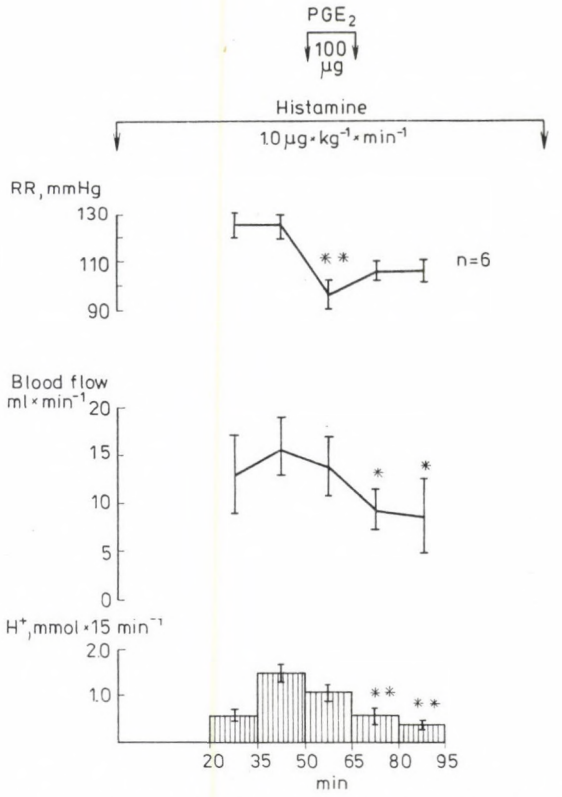



*Fig. 2.* Effect of PG-$E_2$ on histamine stimulated gastric mucosa; n = number of dogs. Top: systemic blood pressure, Middle: aminopyrine clearance (MBF). Bottom: Acid secretion in 15 min periods; mean ± S.D. * = $P < 0.05$ vs 1st period, ** = $P < 0.01$ vs 1st period. Arrows indicate the duration of histamine and PG-$E_2$ infusion

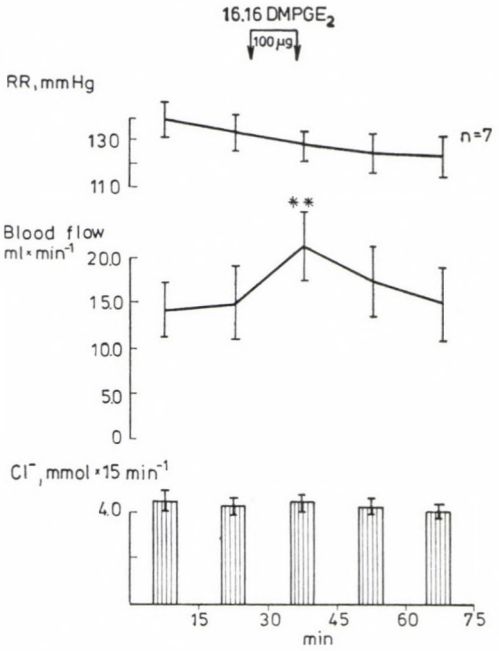



*Fig. 3.* Effect of 16-16-DMPGE$_2$ on resting gastric mucosa. n = number of dogs. Top: systemic blood pressure, Middle: aminopyrine clearance (MBF). Bottom: chloride secretion in 15 min periods; mean ± S.D. ** = $P < 0.01$ vs 1st period. Arrows indicate the duration of 16-16-DMPGE$_2$ infusion

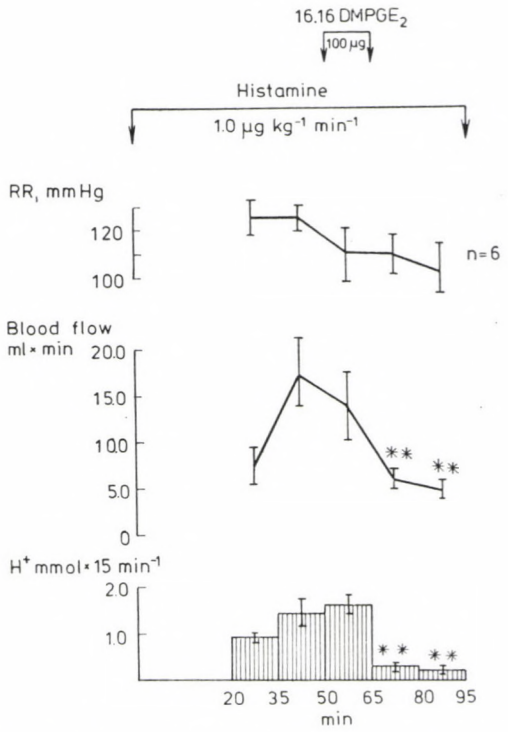



*Fig. 4.* Effect of 16-16-DMPGE$_2$ on histamine stimulated gastric mucosa. n = number of dogs Top: systemic blood pressure, Middle: aminopyrine clearance (MBF), Bottom: acid secretion in 15 min periods. mean ± S.D. ** = $P < 0.01$ vs 1st period. Arrows indicate the duration of histamine and 16-16-DMPGE$_2$ infusion

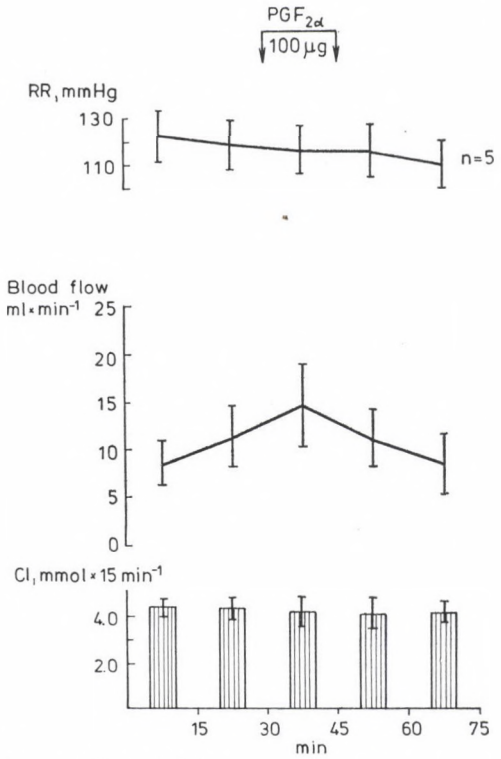



*Fig. 5.* Effect of PG-$F_{2\alpha}$ on resting gastric mucosa n = number of dogs. Top: systemic blood pressure Middle: aminopyrine clearance (MBF) Bottom: chloride secretion in 15 min periods; mean ± S.D. Arrows indicate the duration of PG-$F_{2\alpha}$ infusion

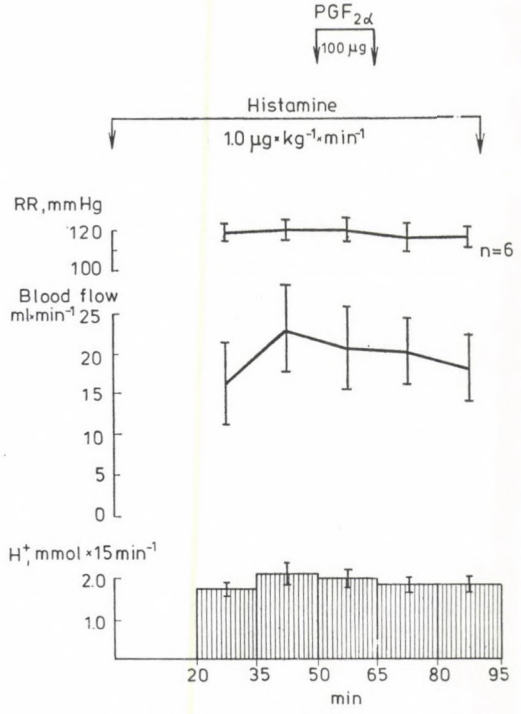



*Fig. 6.* Effect of PG-$E_{2\alpha}$ on histamine stimulated gastric mucosa. n = number of dogs. Top: systemic blood pressure Middle: aminopyrine clearance (MBF) Bottom: acid secretion in 15 min periods; mean ± S.D. Arrows indicate the duration of histamine and PG-$F_{2\alpha}$ infusion

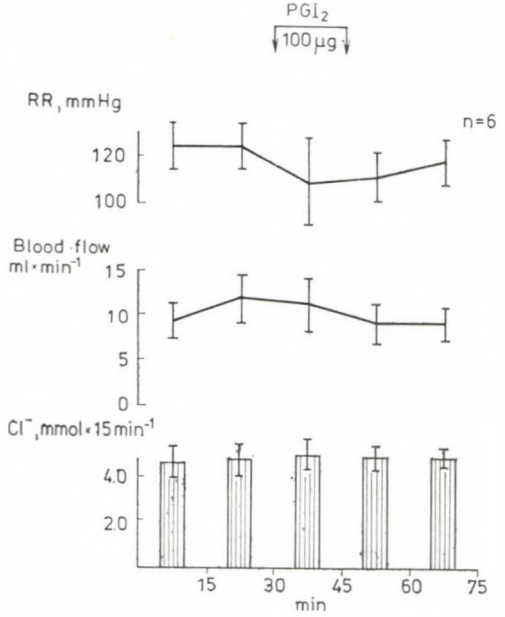



*Fig. 7.* Effect of PG-$I_2$ on resting gastric mucosa n = number of dogs. Top: systemic blood pressure Middle: aminopyrine clearance (MBF) Bottom: chloride secretion in 15 min periods; mean ± S.D. Arrows indicate the duration of PG-$I_2$ infusion

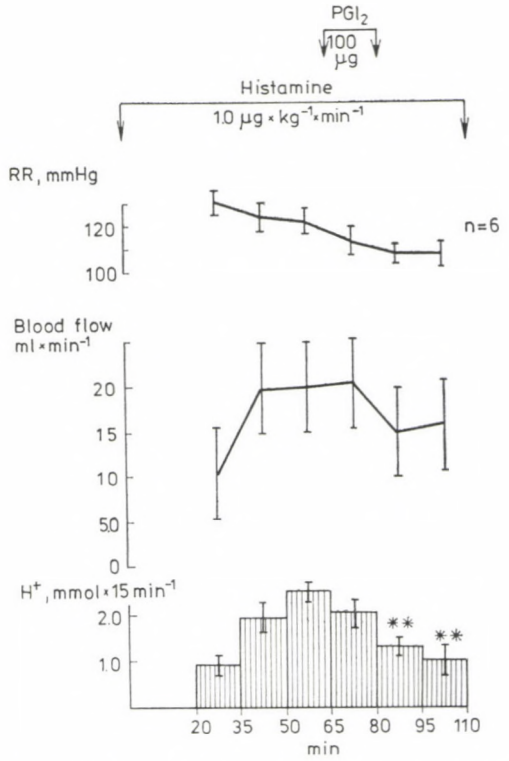



*Fig. 8.* Effect of PG-$I_2$ on histamine stimulated gastric mucosa, n = number of dogs. Top: systemic blood pressure, Middle: aminopyrine clearance (MBF), Bottom: acid secretion in 15 min periods. mean ± S.D. ** = $P < 0.01$ vs 1st period. Arrows indicate the duration of histamine and PG-$I_2$ infusion

Since basal acid secretion is negligible in the canine stomach, the aminopyrine clearance in experiments with PGs given without histamine (resting mucosa) was measured by introducing an 0.1 N HCl solution into the stomach and recovering it at 15 min intervals.

Stimulation of acid secretion was done by an intravenous infusion of histamine in a dose of 1.0 $\mu$g/kg/min through a femoral vein.

The PGs tested were: PG-$I_2$, PG-$E_2$, 16-16-DMPG$E_2$ and PG-$F_{2\alpha}$.

A stock solution of PG-$E_2$, 16-16-DMPG$E_2$ and PG-$F_{2\alpha}$ was made up freshly (1 mg/ml) in 0.02% ice-cold sodium-carbonate solution. PG-$I_2$ was dissolved (1 mg/ml) freshly in 0.05 M Tris-buffer pH 9.6. Dilutions were made immediately before use in ice-cold isotonic sodium bicarbonate solution, and injected intraarterially in a single dose of 100 $\mu$g, using a peristaltic pump, during a 15 min period.

All values reported here are mean $\pm$ S.D. and differences were considered significant if the value calculated from Student's *t*-test was less than 0.05.

## Results

The experimental results are presented in Figs 1–8 and in Table I.

**Table I**

*Effect of different prostaglandins on the histamine stimulated gastric mucosa*

| Group | R-value (mean $\pm$ SD) |
|---|---|
| Control | 3.12 $\pm$ 0.64 |
| PG-$E_2$ | 3.88 $\pm$ 0.39 |
| 16-16-DMPG$E_2$ | 3.92 $\pm$ 0.41 |
| PG-$F_{2\alpha}$ | 3.24 $\pm$ 0.58 |
| PG-$I_2$ | 6.79 $\pm$ 0.82* |

* = $P < 0.05$ vs Control

## Discussion

The majority of the investigated PG-analogues are vasoactive substances. According to previous data PG-$F_{2\alpha}$ causes mesenteric vasoconstriction in dogs while PG-$E_2$ and 16-16-DMPG$E_2$ increase blood flow and decrease vascular resistance in the mesenteric vessels [4, 6]. Main and Whittle [7] found that PG-$E_2$ increased the resting blood flow in the gastric mucosa.

On the basis of our investigations it seems that in the resting mucosa of the dog in contrast to the observation of Whittle et al. [11] in the rat, PG-$F_{2\alpha}$ had no vasoconstrictive effect and PG-$I_2$ in the dose administered, did not increase blood flow.

Aminopyrine clearance showed a significant elevation during PG-$E_2$ and 16-16-DMPG$E_2$ treatment without a concomitant rise in basal acid secretion. These results are in agreement with the data of Robert et al. [9] concerning the lack of an antisecretory effect of PG-$F_{2\alpha}$.

In the histamine stimulated gastric mucosa, treatment with PG-$E_2$ or 16-16-DMPG$E_2$ resulted in a strong fall of secretion and of MBF.

PG-$I_2$ inhibited gastric acid secretion but did not cause a substantial change of MBF. This finding is in contrast with the report of Whittle et al. [11].

PG-$F_{2\alpha}$ did not affect $H^+$ secretion and MBF.

The R-value increased significantly in the case of PG-$I_2$ treatment, so that this PG-analogue seemed to have a direct effect on gastric parietal cells. The strong antisecretory effect and the well-known cytoprotective property of this material [2] strengthen the possibility of its use in the therapy of ulcer disease.

## REFERENCES


1. Baker, R., Jaffe, B. M., Reed, J. D., Shaw, B., Venables, C. W.: Endogenous prostaglandins and gastric secretion in the cat. J. Physiol. (Lond.) **278,** 451–460 (1978).
2. Bálint, G. A., Varró, V.: Actinomycin inhibits gastric mucosal protection by prostacyclin in rats. Prostaglandins **21,** 255–257 (1981).
3. Brodie, B. D., Axelrod, J.: The fate of aminopyrine (Pyramidon) in man and methods for the estimation of aminopyrine and its metabolites in biological materials. J. Pharmacol. exp. Ther. **99,** 171–184 (1950).
4. Chapnick, B. M., Feigen, L. P., Hyman, A. L., Kadowitz, P. J.: Differential effects of prostaglandins in the mesenteric vascular bed. Amer. J. Physiol. **235,** H326–H332 (1978).
5. Jacobson, E. D., Linford, R. H., Grossman, M. I.: Gastric secretion in relation to mucosal blood flow studied by a clearance technic. J. Clin. Invest. **45,** 1–13 (1966).
6. Kowalewski, K., Kolodej, A.: Effect of 16,16-dimethyl-$PGE_2$ given intra-arterially or intra-gastrically, on acid secretion by canine stomachs perfused ex vivo. Prostaglandins **15,** 901–906 (1978).
7. Main, I. H. M., Whittle, B. J. R.: The effects of E and A prostaglandins on gastric mucosal blood flow and acid secretion in the rat. Brit. J. Pharmacol. **49,** 428–436 (1973).
8. Moncada, S., Salmon, J. A., Vane, J. R., Whittle, B. J. R.: Formation of prostacyclin and its product, 6-oxo-PG-$F_{1\alpha}$ by the gastric mucosa of several species. J. Physiol. (Lond.) **275,** 4–5P (1978).
9. Robert, A., Nezamis, J. E., Phillips, J. P.: Inhibition of gastric secretion by prostaglandins. Amer. J. Digest. Dis. **12,** 1073–1076 (1967).
10. Varró, V., Döbrönte, Z., Sági, I.: Interrelation between gastric blood flow and HCl secretion in dogs. Acta med. Acad. Sci. hung. **35,** 1–20 (1978).
11. Whittle, B. J. R., Boughton-Smith, N. K., Moncada, S., Vane, J. R.: Actions of prostacyclin (PG-$I_2$) and its product 6-oxo-PG-$F_{1\alpha}$ in the rat gastric mucosa in vivo and in vitro. Prostaglandins **15,** 955–967 (1978).

*Acta Medica Hungarica, 41 (2—3), pp. 157—161 (1984)*

## *Nutrition*

# EXPERIMENTAL STUDY OF THE NUTRITIONAL BIOLOGICAL CHARACTERS OF FERMENTED MILKS

A. Vass, S. Szakály, P. Schmidt[1]

HUNGARIAN INSTITUTE FOR DAIRY RESEARCH AND [1]INSTITUTE OF PATHOPHYSIOLOGY, UNIVERSITY MEDICAL SCHOOL, PÉCS, HUNGARY

Received: June 11, 1982

Fermented milks (yogurt, kefir, sour milk), compared with milk for their non-protein nitrogen and free amino-nitrogen contents gave higher values prior to as well as 1, 2 and 3 h after pepsin digestion in vitro. In feeding trials of 6 weeks duration with diets based on casein, milk and fermented milks, respectively, those groups of weanling (50 to 60 g) male Wistar rats showed the maximum increments of body mass which had been fed the diets based on fermented milks. The highest values of the protein utilization index (increase in body mass in g per protein intake in g) were found in the animals kept on the fermented milk products. The favourable protein utilization and body mass increment on fermented milk diets are attributed to a better digestibility of proteins in these products.

**Keywords:** fermented milks, protein utilization, protein digestion, milk diets

## Introduction

The microbiological and biochemical processes associated with the processing of fermented milks favourably modify the biological value of milk [1] This applies primarily to the vitamin concentrations and to the proteins.

In the stage of rapid growth the bacteria utilize vitamins A, $B_1$, $B_2$, $B_{12}$ vitamins and pantothenic acid, while forming vitamin $B_1$, pyridoxine, niacin and folic acid in large amounts [3].

In the course of fermentation 1 to 2% of the proteins are degraded. Rasic et al. [6], in a comparative study of the quantitative distribution of amino-acids in different kinds of yogurt from milk of cows and sheep found that fermentation adds to the biological value of milk.

According to Breslaw and Kleyn [2], protein in yogurts is superior in digestibility to milk protein. Simhaee and Keshavarz [8], using dried yogurt and dried milk for the nutrition of children, found dried yogurt of higher biological value. Sandine et al. [7] point to the benefits of lyophilized, fermented milk concentrates in enteral diseases.

Send offprint requests to A. Vass, Hungarian Institute for Dairy Research, H-7623 Pécs, Tüzér u. 15, Hungary

Though the milk fats are little affected by the process of fermentation, their digestibility is better in fermented than in non-fermented milks [2]. Their degradation products belong to the essential flavouring factors of these products.

On the evidence of these studies, the nutritional properties of fermented milks are well established in many respects. No such observations concerning milks of Hungarian source have been published so far. It seemed therefore justified to undertake a comprehensive study of these products, so as to examine their biological value, on the one hand, and to provide a basis for the development and large-scale production of milks of this kind, on the other. The first step was the study of currently used fermented milks for the digestibility and utilization of their proteins.

## Material and methods

Milk containing 3.6% fat, and yogurt, kefir and sour milk prepared therefrom were examined, the products being incubated subsequent to fermentation at 5 °C for 24 h. For the feeding trials the products were lyophilized and the rat feeds were prepared. The composition of the diets is given in Table I.

**Table I**

*Composition of diets in percentage*

| | Based on | |
|---|---|---|
| | Milk products, g | Casein, g |
| Lyophilizates (milk, yogurt, kefir, sour milk) | 46 | — |
| Casein | — | 15 |
| Starch | 43,5 | 61,5 |
| Sunflower-oil | — | 13 |
| Lucerne fibre | 6 | 6 |
| Vitamin premix | 1 | 1 |
| AP-17 | 3 | 3 |
| NaCl | 0.5 | 0.5 |
| Total | 100 | 100 |

The milk products were examined for digestibility in vitro by the use of pepsin according to Breslaw and Kleyn [2]. The hourly samples were measured spectrophotometrically for non-protein nitrogen (NPN) and free aminonitrogen (FAN) [9] after precipitation of the proteins with sulphosalicylic acid [4].

For the feeding tests weanling male Wistar rats of 50 to 60 g body mass were used. They received the individual diets by groups of five for 6 weeks ad libitum, parallel with measurements of body mass. Each food was tested on 2 groups i.e. on 10 animals. From the increment of body mass found at the end of 6 weeks the protein utilization index (body mass increment g per total protein intake g) was calculated.

## Results

Figure 1 shows the NPN concentrations, Figure 2 the FAN concentrations of milk and fermented milks in the various stages of pepsin digestion.

Both diagrams clearly show that the fermented milks differ little from each other, but contain more NPN and FAN than does milk, either before or in the course of digestion. The mean NPN and FAN values of fermented milks differ significantly from those of milk.

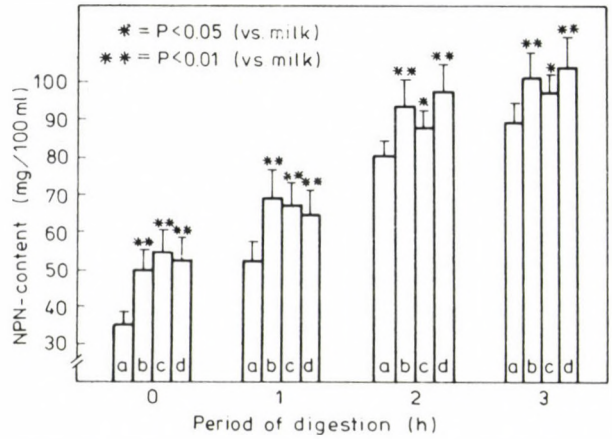



*Fig. 1.* NPN concentration in milk (a), yogurt (b), kefir (c) and sour milk (d) in the successive stages of pepsin digestion; mean ± S.D., n = 5 in each group

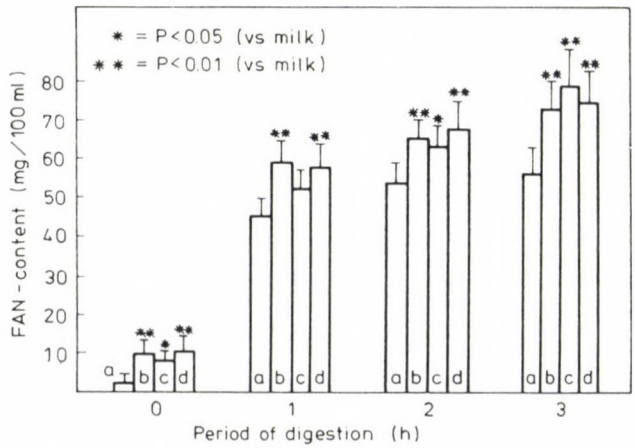



*Fig. 2.* FAN concentration in milk (a), yogurt (b), kefir (c) and sour milk (d) in the successive stages of pepsin digestion; mean ± S.D., n = 5 in each group

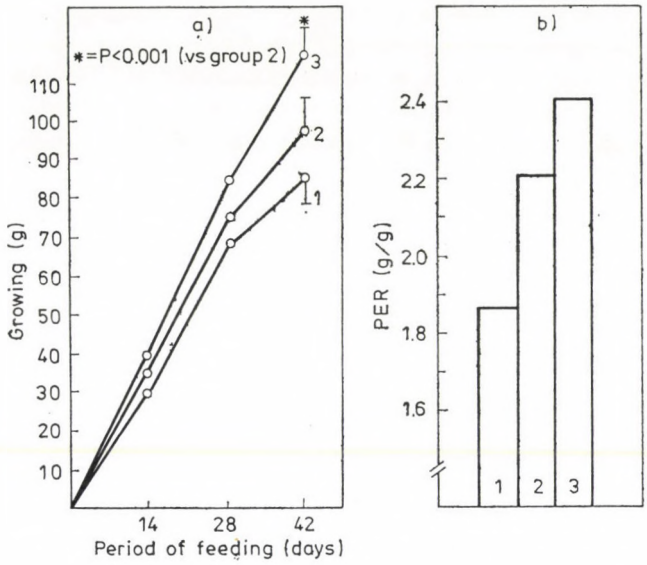



*Fig. 3. a* Gain in body mass of weanling rats fed diets based on casein (1), milk (2) and fermented milks (3); mean ± S.D., n = 10 in each group. *b* Protein utilization index calculated from the 42 day values (n = 10 for each group)

Figure 3 represents the body mass increments found at 2, 4 and 6 weeks of feeding (diagram "a"), but for the fermented milks only the mean values are given, the difference between the values of the individual products being negligible. Diagram "b" shows the protein utilization values of the individual diets based on casein, milk and fermented milks, respectively (Fig. 3).

From the diagrams it clearly emerges that the biological value of the foods under study increases in the order: casein, milk, fermented milks.

## Discussion

The present results are consistent with published evidence, according to which the fermented milks are superior in biological value to milk from which they are prepared. The composition of the foods allowed to eliminate possible factors (e.g. loose stools) interfering with the accuracy of the results [3]. It could be thus ascertained beyond doubt that fermented milks permit a better utilization of protein than do milk-based foods of the same protein content.

One of the causes of the high utilization of protein from fermented milks lies in its digestibility (Figs 1, 2), which may be connected in all likelihood with the particular structure of the curd [5] and with partial degradation of the proteins [6] produced by the lactobacilli.

## REFERENCES

1. Blanc, B.: Der Wert der Sauermilchprodukte in der modernen Ernährung I., II. Dtsch. Milchwirtsch. **25,** 1030–1032; 1056–1061 (1974).
2. Breslaw, E. S., Kleyn, D. H.: In vitro digestibility of protein in yogurt at various stages of processing. J. Food Sci. **38,** 1016–1021 (1973).
3. Hargrove, R. E., Alford, J. A.: Growth rate and feed efficiency of rats fed yogurt and other fermented milks. J. Dairy Sci., **61,** 11–19 (1978).
4. Hinsberg, K., Lang, K.: Medizinische Chemie für den klinischen und theoretischen Gebrauch. Urban und Schwarzenberg, München—Berlin—Wien 1957.
5. Miller, J., Kandler, O.: Untersuchungen über den Eiweissabbau in Sauermilchen. Med. Ernähr. **5,** 100–107 (1964).
6. Rasic, J., Stojsavljevic, T., Curcic, R.: A study of the amino acids of yogurt. II. Amino acid content and biological value of protein of different kinds of yogurt. Milchwissenschaft **26,** 219–224 (1971)
7. Sandine, W. E., Muralidhara, K. S., Elliker, P. R., England, D. C.: Lactic acid bacteria in food and health: A review with special reference to enteropathogenic Escherichia coli as well as certain enteric diseases and their treatment with antibiotics and lactobacilli. J. Milk Food Techn., **35,** 691–702 (1972).
8. Simhaee, E., Keshavarz, E.: Comparison of gross protein value and metabolizable energy of dried skim milk and dried yogurt. Poult. Sci. **53,** 184–191 (1974).
9. Wolfschoon-Pombo, A. F., Klostermeyer, H., Weiss, G.: Die NPN-Fraktion der Kuhmilch. V. Analytik. Milchwissenschaft **37,** 80–85 (1982).

*Acta Medica Hungarica, 41 (2—3), pp. 163—169 (1984)*

# EFFECT OF FERMENTED MILK DIETS ON REGENERATION OF THE RAT LIVER

P. SCHMIDT, A. VASS[1], S. SZAKÁLY[1]

INSTITUTE OF PATHOPHYSIOLOGY, UNIVERSITY MEDICAL SCHOOL, PÉCS AND [1]HUNGARIAN INSTITUTE OF DAIRY RESEARCH, PÉCS, HUNGARY

Received: June 11, 1982

In a comparative study of milk versus fermented milk (yogurt, kefir, sour milk) diets concerning their effect on body mass and on liver regeneration in partially (70%) hepatectomized male CFY rats significantly higher values were found three weeks after the operation in the groups fed on fermented milk diets than in those fed on milk-based diets. No differences between the chemical components of the regenerated liver (solids, total lipids, total N) were found either between the individual groups, or between the various groups and the control. The regenerated liver corresponded to the normal organ in every respect.

The values reflecting the increments of solids, total lipids and total N of the regenerating liver, whether expressed in absolute figures or in per cents, were found to be higher in the animals fed fermented milk diets than in those fed milk-based diets.

The invention that the biochemical changes associated with bacterial fermentation of milk are beneficial to liver regeneration provides new evidence in support of the high biological nutritive value of fermentation.

**Keywords:** fermented milk diets, regeneration of liver, biological nutritive value, rat

## Introduction

The nutritive value of fermented milks is amply documented in the literature. According to Rasic et al. [12], the proteins of yogurt are superior in nutritive quality to those of milk. Yogurt has been found by Simhaee and Keshavarz [13] to provide for a higher growth rate in chicks than does milk. Similar results have been obtained by Hargrove and Alford in rats [7].

As it is known after partial hepatectomy (70 to 75%) the original mass of the liver regenerates fully in a few weeks, without any particular damage. The DNA-synthesizing capacity of the liver cells is related to the regeneration of the organ [11], thus reflecting an enhanced protein synthesis. The chemical components of the regenerating liver have also been widely studied. The regenerating liver has been examined one to two weeks after partial hepatectomy for water content by Higgins and Anderson [8], for nitrogen content by Brues et al. [2], for lipids by Ludewig et al. [9]. Regeneration is greatly

Send offprint requests to P. Schmidt, Institute of Pathophysiology, University Medical School, H-7643 Pécs, Szigeti út 12, Hungary

affected by the composition of food. The liver of exclusively glucose-fed animals show a poor regenerative tendency [2]. Handler and Bernheim [6] found in hepatectomized animals fed on a low-calorie diet a reduction in body mass during normal liver regeneration. The mass of the regenerated liver is closely related to the protein content of the diet [10].

Since it has been confirmed by published evidence that nutrition of high biological value intensifies hepatic regeneration, it was deemed of interest to compare milk with fermented milk products as to their effect on liver regeneration.

## Materials and methods

Milk and fermented milks (yogurt, kefir, sour milk) served as a basis for diets containing 3.9% protein, 1.9% fat and 5.7% lactose. The prepared foods consisted of a mixture 2 : 1 of one of the above products and of home-prepared basic feed (7% casein, 82% corn-starch, 6.5% lucerne fibre, 1% LATI vitamin-premix, 3% AP–17 mineral mixture, and 0.5% NaCl). In this manner the feeds were equal in chemical composition (38% solids of which 12% were protein, 5% fat, 75% carbohydrate) and energy content (1687 kJ/100 g solids).

In the study 77 male CFY rats of 200 to 250 g body mass were used.

Over the last 14 days before operation the animals were habituated to the respective diets, but were fasted for the last 16 h. The operation was performed under inhalation anaesthesia with ether. The abdomen was opened by midline incision. The left lateral and the right and left middle lobes of the liver were exposed, drawn apart, then the respective vessels were tied, and the lobes were removed and placed in measuring glasses and immediately weighed. At the end of the three week test period the regenerating and the control (intact) livers were excised and the mass of the organs was determined. The mass of the original liver was calculated from the masses of the resected lobes, according to Gurd et al. [5]. Since the lateral lobe and the middle lobe made up of two lobes account for 69.4% of the total liver mass, the mass of the three resected lobes, multiplied with the factor 1.4110, gives the total liver mass.

The livers of the intact and hepatectomized animals were measured for solids, fats (Soxhletextraction), proteins (Kjeldahl method) and DNA [3] at the end of the period of study and compared with the same components of the lobes found after hepatectomy. The results are expressed as mean $\pm$ S.D.

## Results

In Fig. 1 the body masses of the animals kept on the various milk diets have been plotted against time.

It can be seen that in the three groups fed with diets based on fermented milk the increments of body mass by the end of the feeding trial of three weeks were significantly in excess of those found in the group kept on milk-based diet ($P < 0.01$). The body mass increments, expressed in per cents of the values found at the start of the experiment, were, yogurt, 32%; kefir, 31.6%; sour milk, 33.8% and milk, 23.4%.

Figure 2 shows the increments of liver mass in the various groups, expressed in per cents of the liver mass prior to operation. The liver mass of the animals in the individual groups had been roughly equal at the start of the period of study (these having been calculated from the excised lobes, as described earlier).

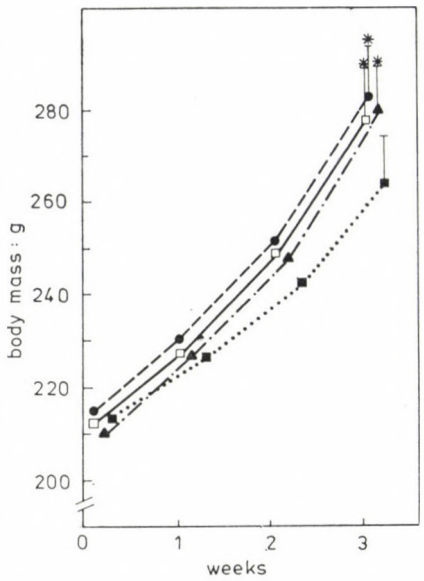



*Fig. 1.* Body mass of animals fed diets based on fermented milk products versus milk, plotted, against the time elapsed since partial hepatectomy
■ .... ■ milk, n = 16; □—□ yogurt, n = 11; ●---● kefir, n = 9; ▲—·—▲ sour milk, n = 6
mean + S.D, $P < 0.01$, referred to the milk-based diet

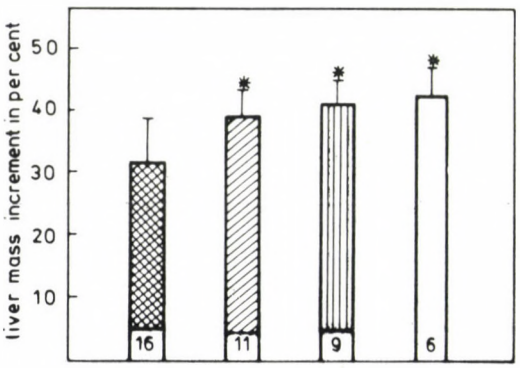



*Fig. 2.* Liver mass increments of animals fed diets based on fermented milk products versus milk, found 3 weeks after partial hepatectomy. ▒ milk ▩ yogurt, ▥ kefir, □ sour milk
mean + S.D., $P < 0.01$ referred to the milk-based diet

The increments of liver mass, similarly to those of body mass, also showed significantly higher values ($P < 0.01$) in the groups fed fermented milks.

**Table I**

*Components of regenerated (R) and intact (I) rat livers*

| Liver components g/100 g fresh liver | Diets | Milk-based | | Yogurt-based | | Kefir-based | | Sour milk-based | |
|---|---|---|---|---|---|---|---|---|---|
| | | R (n = 16) | I (n = 10) | R (n = 11) | I (n = 10) | R (n = 9) | I (n = 9) | R (n = 6) | I (n = 6) |
| Solids | mean | 27.1 | 26.8 | 26.9 | 26.0 | 26.9 | 25.8 | 27.1 | 27.3 |
| | ± S.D. | 1.7 | 1.9 | 2.0 | 1.8 | 1.6 | 1.7 | 1.1 | 1.5 |
| Total N | mean | 3.01 | 2.86 | 3.03 | 2.87 | 2.96 | 2.80 | 2.92 | 3.11 |
| | ± S.D. | 0.25 | 0.30 | 0.20 | 0.19 | 0.34 | 0.24 | 0.30 | 0.33 |
| Total lipids | mean | 5.13 | 4.58 | 5.24 | 4.92 | 4.70 | 4.48 | 4.80 | 4.86 |
| | ± S.D. | 0.64 | 0.55 | 0.68 | 0.48 | 0.39 | 0.44 | 0.46 | 0.52 |

n = number of rats

In Table I the values for solids, total N and total lipids in the regenerating liver removed at the end of the three week feeding period against the control (intact) livers, are given in per cents.

Table II shows the increments of solids, total N and total lipids of the regenerated livers, expressed in absolute values and in terms of per cent referred to the preoperative values.

The figures in Table I show no significant differences between the percentage values of the components of regenerated and intact livers. In other words, the hepatectomized animals fed the diets under study, regenerated livers similar in composition to the intact organ. The values of solids indicate that, in opposition to other observations [4, 14, 15], in the present study the improved regeneration of the liver was not associated with an increase in its water content.

From the figures presented in Table II it clearly emerges that in the animals fed on fermented milk diets the given increments, whether expressed in absolute figures or in terms of per cent, were in excess of the respective values of the animals fed a milk-based diet. The excessive nitrogen values in the groups fed fermented milks point to an enhanced protein synthesis.

The proportion of DNA in the livers of hepatectomized animals was higher than in those of intact animals. This also points to an enhanced protein synthesis and to smaller cell sizes. No significant differences were, however, found between the percentage figures of DNA in the liver of hepatectomized animals kept on different diets (Table III).

**Table II**

*Increments of liver components in partially hepatectomized animals fed diets based on milk and fermented milks*

| Liver components | Diets | Milk-based (n = 16) | | Yogurt-based (n = 11) | | Kefir-based (n = 9) | | Sour milk-based (n = 6) | |
|---|---|---|---|---|---|---|---|---|---|
| | | M | S.D. | M | S.D. | M | S.D. | M | S.D. |
| Increments | g | 0.45 | 0.12 | 0.66** | 0.16 | 0.59* | 0.11 | 0.63* | 0.13 |
| in solids | per cent | 27.1 | 5.1 | 43.1** | 8.6 | 39.2** | 6.1 | 36.2* | 5.6 |
| Increments | g | 0.01 | 0.009 | 0.04** | 0.014 | 0.03** | 0.012 | 0.03** | 0.011 |
| in total N | per cent | 6.3 | 3.2 | 20.0** | 5.5 | 12.1* | 4.5 | 11.4* | 4.3 |
| Increments | g | 0.05 | 0.03 | 0.11** | 0.04 | 0.07 | 0.04 | 0.11* | 0.05 |
| in total lipids | per cent | 16.2 | 12.2 | 39.9** | 14.5 | 19.6 | 11.3 | 34.4* | 13.8 |

* $P < 0.01$, referred to milk-based diet
** $P < 0.001$, referred to milk-based diet
n = number of rats

**Table III**

*DNA- concentration of the regenerated (R) and intact (I) livers*

| | Diets | Milk-based | | Yogurt-based | | Kefir-based | | Sour milk-based | |
|---|---|---|---|---|---|---|---|---|---|
| | | R (n = 16) | I (n = 10) | R (n = 11) | I (n = 10) | R (n = 9) | I (n = 9) | R (n = 6) | I (n = 6) |
| DNA mg/100 g | mean | 10.6* | 9.8 | 10.9* | 9.7 | 11.0* | 9.9 | 10.8* | 9.5 |
| fresh mass | ± S.D. | 0.7 | 0.6 | 0.6 | 0.6 | 0.7 | 0.8 | 0.7 | 0.6 |

* $P < 0.01$, referred to the respective intact group
n = number of rats

## Discussion

The nutritive value of a fermented milk product is generally held to be similar to that of milk from which it is prepared. Fermentation produces a loss of vitamin $B_{12}$ and in yogurts the concentrations of riboflavin, pantothenic acid, thiamine and biotin are reduced. Kefir contains more niacin, thiamine, pyridoxine and folic acid, while yogurt contains more folic acid, than does milk. The lactose concentrations also decrease as a result of its partial conversion to lactic acid. According to published evidence, yogurt proteins are superior in digestibility to milk proteins [1]. It is alleged for the explanation of the higher nutritive value of fermented milks that, as a result of slow fermentation produced by lactobacilli, curd of a fine flocculent structure is formed, moreover, in the course of processing a certain proportion of the proteins is split into peptides and free amino-acids.

In opposition to the diets fed by Hargrove and Alford [7] consisting of milk alone, only two thirds of the diets fed in the present study were made up of milk. This allowed to avoid loose stools in the animals. The differences in pH alone fail to account for the beneficial effects of the fermented milk on body mass increase and on liver regeneration. Actually, Hargrove and Alford, in their feeding trials obtained significantly lower figures with milks acidified directly with lactic acid.

According to the results of Pénzes and Barna [10], the process of regeneration is greatly affected by the proportion of protein in the diet. This emphasizes the importance of dietary protein in regeneration. In the present study the individual protein and amino-acid compositions of milk and fermented milk products may have contributed to the intensity of liver regeneration in the animals fed fermented milk diets, but a possible role of other biochemical factors cannot be ruled out either. It is most unlikely that the vitamin contents of the various products should have played any part in liver regeneration, since, as a result of the effect of the different bacteria the amounts of vitamins in the fermented milks are highly variable, but there were no differences in this respect between the results found in the individual groups. An increase in the absolute nitrogen figures found in the regenerated livers is suggestive of an intensified protein synthesis.

In sum, the present result indicates that the biochemical changes produced by fermentation benefit the process of regeneration. This may be regarded as further evidence in support of the nutritive efficiency of fermentation.

## REFERENCES


1. Breslaw, E. S., Kleyn, D. H.: In vitro digestibility of protein yogurt at various stages of processing. J. Food Sci. **38,** 1016–1021 (1973).
2. Brues, A. M., Drury, D. R., Brues, M. C.: A quantitative study of cell growth in regenerating liver. Arch. Path. **22,** 658–673 (1936).
3. Burton, K.: A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of desoxyribonucleic acid. Biochem. J. **62,** 315–323 (1956).
4. Fazekas, S.: Ép és regeneráló májszövet néhány dehidrogenáze aktivitása és ezek napszakos ingadozása. Thesis, Budapest 1958.
5. Gurd, F. N., Vars, H. M., Ravdin, I. S.: Composition of the regenerating liver after partial hepatectomy in normal and protein depleted rats. Amer. J. Physiol. **152,** 11–21 (1948).
6. Handler, P., Bernheim, F.: The effect of choline deficiency on the fat content of regenerated liver. J. biol. Chem. **148,** 649–654 (1943).
7. Hargrove, R. E., Alford, J. A.: Growth rate and feed efficiency of rats fed yogurt and other fermented milks. J. Dairy Sci., **61,** 11–19 (1977).
8. Higgins, G. M., Anderson, R. M.: Experimental pathology of the liver. I. Restoration of the liver of the white rat following partial surgical removal. Arch. Path. **12,** 186–202 (1931).
9. Ludewig, S., Minor, G. R., Hortenstine, J. C.: The influence of partial hepatectomy on fat content of the regenerating liver. Proc. Soc. exp. Biol. (N. Y.) **42,** 158–159 (1939).
10. Pénzes, L., Barna, J.: Különböző fehérjetartalmú diéták etetésének hatása a patkánymáj regenerációjára. Magy. Tud. Akad. Biol. Oszt. Közl. **5,** 281–293 (1962).
11. Pósalaky, Z., Törő, I., Barka, T.: A nukleinsavak szerepe a májszövet növekedésében in vitro. Kísérl. Orvostud., **5,** 401–410 (1953).
12. Rasic, J., Stojsavljevic, T., Curcic, F.: A study of the amino acids of yogurt. II. Amino acid content and biological value of protein of different kinds of yogurt. Milchwissenschaft **26,** 219–224 (1971).
13. Simhaee, E., Keshavarz, E.: Comparison of gross protein value and metabolizable energy of dried skim milk and dried yogurt. Poult. Sci. **53,** 184–191 (1974).
14. Szego, C. M., Roberts, S.: The influence of ovariectomy on the chemical composition of regenerated rat liver. J. biol. Chem. **178,** 827–838 (1949).
15. Vars, H. M., Gurd, F. N.: The influence of dietary protein upon the regeneration of liver protein in the rat. Fed. Proc **6,** 299 (1947).

*Acta Medica Hungarica, 41 (2—3), pp. 171—173 (1984)*

# USE OF HUNGARIAN MILK-PROTEIN PRODUCTS IN SPORT

R. Frenkl, Ágota Györe

NATIONAL INSTITUTE OF PHYSICAL EDUCATION BUDAPEST, HUNGARY

Received: June 11, 1982

Protein-based food products intended for athletes — (Hamomid powder and tablets, Amino-acid Capsules) — were studied for absorption, elimination and excretion. On the evidence of the findings all were readily absorbed, the serum amino-acid levels attained their peaks 60 to 90 min after ingestion of the daily dose. At the end of 10 day periods during which the products in question had been administered a minor increase was found in the urinary excretion of alpha-aminonitrogen.

**Keywords:** milk-protein products, sport

## Introduction

The competitive sports of our days involving heavy physical training pose major problems of nutrition with adequate protein. In the present study the absorption and elimination of the Hungarian milk protein concentrates Hamomid powder and tablets and Amino-acid Capsules were examined.

## Materials and methods

Composition of the products was as follows.

*Hamomid powder*

| | | | |
|---|---|---|---|
| Protein | 55 % | | |
| Carbohydrates | 20.8% | (lactose 9.5%, glucose 11.3%) | |
| Fats | 19.4% | (coconut butter 12.9%, sunflower oil 6.5%) | |
| Minerals | 2.8% | | |
| Vitamins (mg) | | | |
| A | 0.375 | C | 150.0 |
| $B_1$ | 0.6 | E | 6.0 |
| $B_2$ | 0.9 | biotin | 0.075 |
| $B_6$ | 1.0 | folic acid | 0.1 |
| $B_{12}$ | 0.001 | Ca-panto-thenate | 2.5 |

Hamomid tablets are of the same composition with the difference that they contain no fat.

*Amino-acid Capsules* are casein hydrolysate fortified with tryptophan in 0.4 g capsules.

For the studies of absorption and metabolism, groups of 12 subjects were formed. The following forms of treatment were applied: *1.* Placebo. *2.* Hamomid powder. *3.* Hamomid tablets. *4.* Amino-acid Capsules. Each subject underwent all four forms of treatment, each

Send offprint requests to R. Frenkl, National Institute of Physical Education, H-1123, Budapest, Alkotás u. 44, Hungary

group being subdivided into groups of three members receiving different treatments. On each day of the trial each form of treatment was thus represented. On summarizing the findings no significant differences between the results obtained on the different days were found. The results have therefore been given for the total groups of 12 subjects.

The trial was conducted as follows.

The test subjects reported at 8 o'clock a.m., bringing the first 24 h urine. At 8 o'clock the first blood sample (0 h value) was withdrawn. Then each person received the daily dose of the respective product. Blood samples were taken at 30, 60, 90 and 120 min. α-amino-N was measured in the samples photometrically by the ninhydrin reaction, according to Mütig and Kaiser [3].

Essential steps of the procedure:

The serum samples are deproteinized with an ethanol-aceton mixture (1 : 1) and centrifuged. The supernatant is used for the ninhydrin reaction. The values are read on an Unicam 600 spectrophotometer at 580 $\mu$m, a fresh calibration series being prepared for each measurement.

The same method was used for the measurement of urinary α-amino-N. Urine was collected on the day of the study as well.

Subsequently each person continued on the daily dose of the respective product for nine days and reported for investigation on the 10th day with the 24 h urine of the last day. A blood sample was withdrawn. No treatment was given during the next three days. Then the treatment was repeated in the same manner, but with a different product. The entire duration of the trial thus extended to 8 weeks. The investigations were performed on regular days and at regular hours.

## Results and Discussion

Table I shows the serum α-amino-acid level expressed in mg/100 ml. The maximum increase was found after the administration of Hamomid powder, but absorption of the Hamomid tablets and of the Amino-acid Cap-

**Table I**

*Serum α-amino-acid levels (mg/100 ml) in athletes values are means ± S.E.M.*

| | Placebo | Hamomid powder | Hamomid tablets | Amino-acid Capsules |
|---|---|---|---|---|
| 0' | 2.947 | 2.953 | 3.004 | 2.791 |
| | ± 0.317 | ± 0.184 | ± 0.176 | ± 0.290 |
| 30' | 2.990 | 4.591 | 3.893 | 3.800 |
| | ± 0.292 | ± 0.607 | ± 0.380 | ± 0.377 |
| 60' | 3.080 | 5.768 | 4.772 | 4.493 |
| | ± 0.282 | ± 0.661 | ± 0.369 | ± 0.347 |
| 90' | 3.680 | 4.665 | 4.231 | 4.360 |
| | ± 0.249 | ± 0.607 | ± 0.281 | ± 0.078 |
| 120' | 3.070 | 3.540 | 3.580 | 2.947 |
| | ± 0.271 | ± 0.264 | ± 0.287 | ± 0.283 |
| day 10 | 2.853 | 2.589 | 3.111 | 2.613 |
| | ± 0.172 | ± 0.199 | ± 0.285 | ± 0.196 |

**Table II**

*Urinary α-amino-N outputs (mg/24 h) in athletes values are means ± S.E.M.*

| | Placebo | Hamomid powder | Hamomid tablets | Amino-acid Capsules |
|---|---|---|---|---|
| 0' | 143.88 | 149.32 | 159.77 | 145.37 |
| | ± 10.10 | ± 25.76 | ± 20.30 | ± 28.28 |
| 1' | 109.60 | 161.94 | 153.12 | 193.97 |
| | ± 14.16 | ± 22.04 | ± 18.65 | ± 16.63 |
| 10' | 110.71 | 185.53 | 215.90 | 180.05 |
| | ± 15.73 | ± 16.05 | ± 14.04 | ± 15.77 |

sules was also adequate, the peaks having been attained between 60 and 90 min.

Table II shows the urinary values (mg/24 h urine).

Compared with the placebo group, all groups revealed higher values after administration of the protein products, particularly in the urine collected on the 9th day.

On the evidence of the findings, the products under study are readily absorbed and distinctly raise the serum amino-acid level. The insignificant elevation after the use of placebo was due to the fact that the placebo was taken in cocoa.

In agreement with our earlier findings, the amino-acid values are at the lower limit of the normal range (0' value). This is also valid for the urinary amino-acid concentrations. These figures provide a further indirect proof of the necessity of increased protein and amino-acid supplies to athletes. Despite the excess protein intake the serum values at the end of each period were not higher than at the start. Administration of the products is thus within the physiological requirements and the possibilities of regulation. Urinary excretion showed a certain increase, as a sign that an effect of some degree has been attained.

## REFERENCES

1. Frenkl, R.: Anpassungsvorgänge beim trainierten Organismus. Öst. J. Sportmed. **5,** 9–14 (1978).
2. Frenkl, R., Á. Györe: A study of the effect of dietary factors and various exercises on the swimming performance of the albino rat. Physiol. bohemoslov. **28,** 161–168 (1979).
3. Mütig, D., E. Kaiser: Zur quantitativen Bestimmung von α-Amino-Stickstoff in biologischem Material mittels der Ninhydrin-Reaktion. Hoppe-Seylers Z. Physiol. Chem. **332,** 276–279 (1963).

PRINTED IN HUNGARY
Akadémiai Kiadó és Nyomda, Budapest

# INFORMATION FOR AUTHORS

*Acta Medica Hungarica* is published under the auspices of the Hungarian Academy of Sciences. Manuscripts and editorial correspondence should be sent to the editorial office: H-1450 Budapest 9, P. O. Box 67.

Original articles dealing with clinical and experimental medicine will be accepted with the understanding that they have not been and will not be published elsewhere and are subject to editorial revision.

*Form of manuscripts*

Two copies of the manuscript typewritten double-spaced with margins at least 4 cm wide should be submitted. Pages should be numbered consecutively. The first page should contain (1) the title of the paper (2) the initials and first name(s) of the author(s), (3) name of the institution where the work was done, (4) name and address of the author to whom correspondence and offprint requests should be addressed — this will appear as a footnote; (5) an abstract not exceeding 250 works which states the purposes of the study, the main findings and principal conclusions. Below the abstract provide 3 to 10 keywords that will assist indexers in cross-indexing the article.

The text of the paper should be divided into sections with the headings: Introduction, Materials (Patients) and Methods, Results, Discussion, References.

Unusual abbreviations should be identified in an alphabetical list typed after the abstract and keywords.

Drugs must be referred to by their WHO code designation (Recommended International Nonproprietary Names); use of proprietary names is unacceptable.

The international system of units (SI) should be used for all measurements.

*References*

These should be cited in the text as numbers in square brackets. The list of references should contain in alphabetical order of the first authors' names the following: authors' last names with initials; for journal articles the title of the paper (lower case), journal title abbreviated according to the style used in Index Medicus, volume number, inclusive page numbers, year of publication in parentheses; for books the title (upper and lower case), publisher, place and date of publication. Only manuscripts accepted for publication may be included in the reference list.

Examples:

1. Stagg, B. H., Temperly, J. M., Wyllie, J. H.: The fate of pentagastrin. Gut **12**, 825—829 (1971)
2. Falkner, F.: Prevention in Childhood of Health Problems and Adult Life. WHO, Geneva 1980.
3. Fishman, A. P.: Dynamics of pulmonary circulation. In: Hamilton, W. F., Dow, P. (eds): Handbook of Physiology. American Physiological Society, Washington 1963, pp. 65—79.

*Tables*

Each table should be typed on a separate sheet. They should be numbered consecutively with Roman numerals and have a brief specific title. The data presented in the table must be logically and clearly organized and should be self-explanatory. Omit internal horizontal and vertical rules. Cite each table in the text and indicate its approximate place on the margin.

*Illustrations*

Figures should be submitted in duplicate. They must be numbered consecutively with arabic numerals. All figures should bear the name of the first author, the figure number and an arrow indicating the top. Cite each figure in the text and indicate its approximate place on the margin. If a figure has been published, acknowledge the original source and submit written permission from the copyright holder to reproduce the material. Figure captions should be submitted typed double-spaced on a separate sheet.

*Proofs and reprints*

The first authors will receive (1) comments and suggestions of the Editorial Board for improving their paper; (2) a set of proofs for correction; corrected proofs should be returned without delay to the editorial office; (3) 100 reprints free of charge.

## Periodicals of the Hungarian Academy of Sciences are obtainable at the following addresses:

**AUSTRALIA**
C.B.D. LIBRARY AND SUBSCRIPTION SERVICE
Box 4886, G.P.O., *Sydney N.S.W. 2001*
COSMOS BOOKSHOP, 145 Ackland Street
*St. Kilda (Melbourne), Victoria 3182*

**AUSTRIA**
GLOBUS, Höchstädtplatz 3, *1206 Wien XX*

**BELGIUM**
OFFICE INTERNATIONAL DE LIBRAIRIE
30 Avenue Marnix, *1050 Bruxelles*
LIBRAIRIE DU MONDE ENTIER
162 rue du Midi, *1000 Bruxelles*

**BULGARIA**
HEMUS, Bulvar Ruszki 6, *Sofia*

**CANADA**
PANNONIA BOOKS, P.O. Box 1017
Postal Station "B", *Toronto, Ontario M5T 2T8*

**CHINA**
CNPICOR, Periodical Department, P.O. Box 50
*Peking*

**CZECHOSLOVAKIA**
MAD'ARSKÁ KULTURA, Národní třida 22
*115 66 Praha*
PNS DOVOZ TISKU, Vinohradská 46, *Praha 2*
PNS DOVOZ TLAČE, *Bratislava 2*

**DENMARK**
EJNAR MUNKSGAARD, Norregade 6
*1165 Copenhagen K*

**FEDERAL REPUBLIC OF GERMANY**
KUNST UND WISSEN ERICH BIEBER
Postfach 46, *7000 Stuttgart 1*

**FINLAND**
AKATEEMINEN KIRJAKAUPPA, P.O. Box 128
*SF-00101 Helsinki 10*

**FRANCE**
DAWSON-FRANCE S. A., B. P. 40, *91121 Palaiseau*
EUROPÉRIODIQUES S. A., 31 Avenue de Versailles, *78170 La Celle St. Cloud*
OFFICE INTERNATIONAL DE DOCUMENTATION ET LIBRAIRIE, 48 rue Gay-Lussac
*75240 Paris Cedex 05*

**GERMAN DEMOCRATIC REPUBLIC**
HAUS DER UNGARISCHEN KULTUR
Karl Liebknecht-Straße 9, *DDR-102 Berlin*
DEUTSCHE POST ZEITUNGSVERTRIEBSAMT
Sraße der Pariser Kommüne 3–4, *DDR-104 Berlin*

**GREAT BRITAIN**
BLACKWELL'S PERIODICALS DIVISION
Hythe Bridge Street, *Oxford OX1 2ET*
BUMPUS, HALDANE AND MAXWELL LTD.
Cowper Works, *Olney, Bucks MK46 4BN*
COLLET'S HOLDINGS LTD., Denington Estate
*Wellingborough, Northants NN8 2QT*
WM. DAWSON AND SONS LTD., Cannon House
*Folkstone, Kent CT19 5EE*
H. K. LEWIS AND CO., 136 Gower Street
*London WC1E 6BS*

**GREECE**
KOSTARAKIS BROTHERS INTERNATIONAL BOOKSELLERS, 2 Hippokratous Street, *Athens-143*

**HOLLAND**
MEULENHOFF-BRUNA B.V., Beulingstraat 2,
*Amsterdam*
MARTINUS NIJHOFF B.V.
Lange Voorhout 9–11, *Den Haag*
SWETS SUBSCRIPTION SERVICE
347b Heereweg, *Lisse*

**INDIA**
ALLIED PUBLISHING PRIVATE LTD., 13/14
Asaf Ali Road, *New Delhi 110001*
150 B-6 Mount Road, *Madras 600002*
INTERNATIONAL BOOK HOUSE PVT. LTD.
Madame Cama Road, *Bombay 400039*
THE STATE TRADING CORPORATION OF INDIA LTD., Books Import Division, Chandralok
36 Janpath, *New Delhi 110001*

**ITALY**
INTERSCIENTIA, Via Mazzé 28, *10149 Torino*
LIBRERIA COMMISSIONARIA SANSONI, Via Lamarmora 45, *50121 Firenze*
SANTO VANASIA, Via M. Macchi 58
*20124 Milano*
D. E. A., Via Lima 28, *00198 Roma*

**JAPAN**
KINOKUNIYA BOOK-STORE CO. LTD.
17-7 Shinjuku 3 chome, Shinjuku-ku, *Tokyo 160-91*
MARUZEN COMPANY LTD., Book Department,
P.O. Box 5050 Tokyo International, *Tokyo 100-31*
NAUKA LTD. IMPORT DEPARTMENT
2-30-19 Minami Ikebukuro, Toshima-ku, *Tokyo 171*

**KOREA**
CHULPANMUL, *Phenjan*

**NORWAY**
TANUM-TIDSKRIFT-SENTRALEN A.S., Karl Johansgatan 41–43, *1000 Oslo*

**POLAND**
WĘGIERSKI INSTYTUT KULTURY, Marszałkowska 80, *00-517 Warszawa*
CKP I W, ul. Towarowa 28, *00-958 Warszawa*

**ROUMANIA**
D. E. P., *Bucureşti*
ILEXIM, Calea Grivitei 64–66, *Bucureşti*

**SOVIET UNION**
SOJUZPECHAT — IMPORT, *Moscow*
and the post offices in each town
MEZHDUNARODNAYA KNIGA, *Moscow G-200*

**SPAIN**
DIAZ DE SANTOS, Lagasca 95, *Madrid 6*

**SWEDEN**
ALMQVIST AND WIKSELL, Gamla Brogatan 26
*101 20 Stockholm*
GUMPERTS UNIVERSITETSBOKHANDEL AB
Box 346, *401 25 Göteborg 1*

**SWITZERLAND**
KARGER LIBRI AG, Petersgraben 31, *4011 Basel*

**USA**
EBSCO SUBSCRIPTION SERVICES
P.O. Box 1943, *Birmingham, Alabama 35201*
F. W. FAXON COMPANY, INC.
15 Southwest Park, *Westwood Mass. 02090*
THE MOORE-COTTRELL SUBSCRIPTION AGENCIES, *North Cohocton, N. Y. 14868*
READ-MORE PUBLICATIONS, INC.
140 Cedar Street, *New York, N. Y. 10006*
STECHERT-MACMILLAN, INC.
7250 Westfield Avenue, *Pennsauken N. J. 08110*

**YUGOSLAVIA**
JUGOSLOVENSKA KNJIGA, Terazije 27, *Beograd*
FORUM, Vojvode Mišića 1, *21000 Novi Sad*

**Index: 26.015**

# Acta Medica Hungarica

VOLUME 41, NUMBER 4, 1984



Akadémiai Kiadó, Budapest
ACTA MED. HUNG. 41(4) 175—318 (1984) HU ISSN 0236-5286

# ACTA MEDICA HUNGARICA

A QUARTERLY OF THE HUNGARIAN ACADEMY OF SCIENCES

*Acta Medica* publishes reviews and original papers on clinical and experimental medicine in English.

*Acta Medica* is published in yearly volumes of four issues by

AKADÉMIAI KIADÓ

Publishing House of the Hungarian Academy of Sciences
H-1054 Budapest, Alkotmány u. 21

Manuscripts and editorial correspondence should be addressed to the Managing Editor:

Dr. Miklós Papp
*Acta Medica*
H-1083 Budapest, Szigony u 43 or H-1450 Budapest 9, P. O. Box 67

*Subscription information*

Orders should be addressed to

KULTURA Foreign Trading Company
H-1389 Budapest, P. O. Box 149

*Acta Medica is indexed in Current Contents*

CONTENTS

*Acta Medica Hungarica 41 (4) pp. 175—183 (1984)*

## *Endocrinology*

# EFFECT OF THYROTROPIN-RELEASING HORMONE AND GONADOTROPIN-RELEASING HORMONE ON SERUM TSH, PRL, hGH, FSH AND LH IN PRIMARY TESTICULAR FAILURE AND IN HYPOGONADOTROPHIC HYPOGONADISM

G. Szilágyi, I. Szabolcs, G. Vydra,* M. Góth, G. Irsy

FIRST DEPARTMENT OF MEDICINE AND *DEPARTMENT OF UROLOGY, POSTGRADUATE MEDICAL SCHOOL, BUDAPEST, HUNGARY

(Received, 1983)

Infertile males with primary testicular failure, 6 with normal (Group I) and 7 with elevated gonadotropin levels (Group II), 9 patients with Klinefelter's syndrome (Group III) and 5 patients with hypogonadotrophic hypogonadism (Group IV) were investigated. Their serum TSH, PRL, hGH, FSH and LH responses to a single bolus of 200 $\mu$g TRH and 100 $\mu$g GnRH were measured and compared to the corresponding values obtained in 8 fertile healthy males of the same age group. The testosterone levels differed from the control only in the last two groups. Neither the basal TSH level nor the $\triangle$TSH differed between the groups. The latter was significant in all groups.

The basal PRL level was similar in each group except in Group II. where the level was low. After TRH–GnRH treatment the PRL level increased significantly in each group but this increase was less in patients with hypogonadotrophic hypogonadism ($9.94 \pm 2.6$ nmol/l) when compared to the patients with primary testicular failure (Groups I, II, III, together, $n = 22$ $17.10 \pm 2.12$ nmol/l $P < 0.05$). The basal levels of hGH and $\triangle$GH did not differ significantly between the groups. Both FSH and LH showed an exaggerated and protracted increase in patients with primary testicular failure with elevated basal gonadotropin level after TRH–GnRH, while in hypogonadotrophic hypogonadism the response was slight. Neither the serum testosterone nor the serum FSH or LH level influenced the response of TSH and hGH to the TRH–GnRH test.

**Keywords:** hypogonadism, TRH test, GnRH test, growth hormone, prolactin, thyroid stimulating hormone, follicle stimulating hormone, luteinizing hormone.

## Introduction

Opinions differ concerning the effect of thyrotropin releasing hormone (TRH) on the serum thyrotropin (TSH) and prolactin (PRL) level in male hypogonadism. LeRoith et al. [19] found an exaggerated TSH response in primary testicular failure but not in castrated males. In Klinefelter's syndrome the response was mostly normal or even decreased [4, 5, 8, 23, 24]. In hypo-

Send offprint requests to G. Szilágyi H-1389 Budapest, P.O. Box 112, Hungary

gonadotrophic hypogonadism an attenuated response was found in females [25] and a mostly normal one (in 6 out of 8 patients) in males [31].

The different reactions were thought to be due to the different serum testosterone [21] or gonadotropin [24] levels. In the present experiment the response of TSH, PRL, human growth hormone (hGH), follicle stimulating hormone (FSH) and luteinizing hormone (LH) to TRH and GnRH was investigated in patients with testicular failure of different origin causing azoospermia and having different initial serum gonadotropin and testosterone levels.

## Patients and methods

Males with infertility and azoospermia and some with inability of ejaculation were grouped as follows. Group I comprised six males who had azoospermia but not complaint of erection. Their secondary sex characteristics, libido and potency were normal and so were the FSH and LH levels (5.0 to 12.0, and 5.0 to 20.0 U/l, respectively), the sex-chromatic were negative. Their age was between 25 and 31 (mean 28.0 years). The 7 patients in Group II aged 25–36 (mean 28.6) years had exactly the same clinical features and laboratory parameters except that their FSH and LH serum levels were above 20.0 U/l. Group III consisted of patients aged 18 to 52 (mean 29.9) years with Klinefelter's syndrome, as proved by the chromatin positive buccal smear, the 47, XXY kariotype and clinically by small testes, gynaecomastia and sparse body hair. Group IV contained 5 patients aged between 21 and 35 (mean 28.7) years with lack of erections and nocturnal emissions, absence of sexual hair, delayed skeletal maturation, and very small testes and penis. Their basal FSH and LH levels were always below 5.0 $\mu$/l.

The controls were 8 males aged between 21 and 32 (mean 26.4) years. None of the patients received hormonal treatmens before the investigation and all of them were euthyroid TRH 200 $\mu$g (Relefact, Hoechst) and GnRH 100 $\mu$g (Institute for Drug Research, Budapest) were given intravenously in a single bolus at 8.00–9.00 a.m. and blood samples were collected by an indwelling cannula before and at 15, 30, 60 and 120 min after the injection.

Commercial RIA-kits were used to determine the serum level of hormones. The Byk-Mallinckrodt RIA kit was used for TSH (normal $<$6.0 mU/l), Biodata–Serono kits for FSH (normal range, 2.5–12.0 $\mu$/l), LH (normal range, 5.0–20.0 U/l), and hGH (normal $<$279 pmol/l) and IRE Prol RIA kit for PRL (normal range, 2–10 nmol/l). The interassay was found to be 9–17% (coefficient of variation) for TSH, 15–25% for FSH, 11–25% for LH, 21–26% for hGH, and 8–26% for PRL. The lower values were found at the higher, the higher values at the lower levels of hormones.

Testosterone was measured by the method of Vermeulen and Verdonck [29] (normal range 9.0–28.0 nmol/l). Chromosome examination was carried out on peripheral leukocyte cultures, according to the technique of Hungerford [15]. All results were expressed as mean $\pm$S.E.M. analysed using unpaired and paired Student's $t$ test. Differences were considered significant at P values $<0.05$.

## Results

The serum testosterone level was normal in each group except the patients with hypogonadotrophic hypogonadism where the level was extremely low. Though the group of patients with Klinefelter's syndrome showed low normal average value, it differed significantly from the control group (Table I). The basal TSH levels were comparable in every group, and so was the TSH peak after TRH. The effect of TRH was shown by the significant increase in TSH in each group (Table I).

**Table I**

*Testosterone level and TSH response to TRH–Gn RH in different types of hypogonadism and in controls (mean ± S.E.M.)*

| | TSH, mU/l | | | ΔTSH | Testosterone, nmol/l |
|---|---|---|---|---|---|
| | 0 min | 30 min | 60 min | | 0 min |
| Controls (n=8) | 2.21 ±1.30 | 8.48* ±1.89 | 6.40* ±1.45 | 6.27* ±1.52 | 17.79 ± 2.49 |
| Primary testicular failure with normal basal gonadotropin level (n=6) | 3.30 ±0.73 | 10.70* ±2.11 | 8.66* ±1.74 | 7.23* ±1.49 | 15.38 ± 3.49 |
| Primary testicular failure with elevated basal gonadotropin level (n=7) | 2.94 ±0.25 | 10.96* ±1.42 | 8.84* ±1.52 | 8.06* ±1.50 | 13.73 ± 2.95 |
| Klinefelter's syndrome (n=9) | 1.40 ±0.38 | 9.21* ±1.45 | 5.57* ±1.06 | 7.81* ±1.34 | 10.07** ± 1.69 |
| Hypogonadotrophic hypogonadism (n=5) | 3.86 ±0.96 | 13.32 ±4.73 | 17.08 ±6.64 | 11.06* ±4.58 | 5.82** ± 1.75 |

n = number of patients
* Compared to basal level (time 0) the differences are significant statistically
** Compared to the controls the differences are significant statistically

The PRL levels after TRH–GnRH showed a significant increase in each group though the increase in the hypogonadotrophic hypogonadism patients (9.94±2.6 nmol/l) was significantly less ($p < 0.05$) than in those with primary testicular failure: Groups I, II and III together, (n=22) 17.10± ±2.12 nmol/l. The 15 min average PRL value in hypogonadotrophic hypogonadism was also less than the corresponding value of the controls. No difference was found between the controls and the groups with primary testicular failure, and the Klinefelter's syndrome group (Table II). The basal hGH level was similar in all the patients including the controls. No significant increase was found after TRH–GnRH load in either group (Table III). A serum hGH increase of more than 233 pmol/l occurred in one of the 5 patients with normogonadotrophic and one of the 6 patients with hypergonadotrophic primary testicular failure, two out of 9 patients with Klinefelter's syndrome, and one out of the 5 patients with hypogonadotrophic hypogonadism, but none among the controls.

The basal FSH and LH levels were different in the groups and the TRH–GnRH loading caused a significant increase in each of them. In patients with hypogonadotrophic hypogonadism the increase was less than in the controls, while in the patients with primary testicular failure and elevated gonadotropin level a pronounced and sustained increase was observed (Tables IV and V).

**Table II**

*PRL response to TRH–GnRH in different types of hypogonadism and in controls (mean ± S.E.M.)*

| | PRL, nmol/l | | | | | ΔPRL |
|---|---|---|---|---|---|---|
| | 0 min | 15 min | 30 min | 60 min | 120 min | |
| Controls (n=7) | 0.45 ±0.12 | 2.40* ±0.41 | 2.10* ±0.28 | 0.94* ±0.11 | 0.38 ±0.05 | 2.06* ±0.42 |
| Primary testicular failure with normal basal gonadotropin level (n=6) | 0.29 ±0.10 | 1.75* ±0.32 | 1.73* ±0.31 | 0.79* ±0.16 | 0.28 ±0.09 | 1.42* ±0.27 |
| Primary testicular failure with elevated basal gonadotropin level (n=7) | 0.14* ±0.02 | 2.40* ±0.57 | 2.44* ±0.58 | 1.22* ±0.33 | 0.43 ±0.14 | 2.31* ±0.58 |
| Klinefelter's syndrome (n=9) | 0.31 ±0.09 | 2.31* ±0.28 | 1.91* ±0.24 | 1.12* ±0.22 | 0.56 ±0.10 | 1.80* ±0.28 |
| Hypogonadotrophic hypogonadism (n=5) | 0.35 ±0.12 | 1.16** ±0.35 | 1.35* ±0.37 | 0.90* ±0.17 | 0.55 ±0.17 | 1.08* ±0.28 |

n = number of patients
* Compared to basal level (time 0) the differences are significant statistically
** Compared to the controls the differences are significant statistically

**Table III**

*HGH response to TRH–Gn RH in different types of hypogonadism and in controls (mean ± S.E.M.)*

| | HGH, pmol/l | | | | | ΔHGH |
|---|---|---|---|---|---|---|
| | 0 min | 15 min | 30 min | 60 min | 120 min | |
| Controls (n=5) | 18.6 ± 6.04 | 35.34 ± 11.16 | 52.08 ± 26.51 | 48.36 ± 22.32 | 17.67 ± 4.19 | 61.38 ± 26.04 |
| Primary testicular failure with normal basal gonadotropin level (n=5) | 39.99 ± 4.65 | 97.65 ± 39.99 | 120.9 ± 66.03 | 214.83 ±179.03 | 85.56 ± 36.74 | 226.92 ±171.59 |
| Primary testicular failure with elevated basal gonadotropin level (n=6) | 33.95 ± 19.53 | 37.2 ± 19.07 | 33.48 ± 12.56 | 57.20 ± 29.30 | 92.07 ± 50.69 | 88.82 ± 50.22 |
| Klinefelter's syndrome (n=9) | 62.31 ± 20.00 | 143.22 ± 98.12 | 245.06 ±182.28 | 168.33 ±118.58 | 75.33 ± 23.72 | 227.85 ±216.23 |
| Hypogonadotrophic hypogonadism (n=5) | 66.50 ± 25.11 | 41.85 ± 9.3 | 27.9 ± 4.65 | 21.86 ± 3.26 | 112.53 ± 79.05 | 65.57 ± 59.52 |

n = number of patients

## Discussion

According to the results the basal TSH and hGH levels did not differ in the patients with hypogonadism, while in patients with an elevated basal gonadotropin level the PRL level was lower. TRH–GnRH caused a similar

**Table IV**

*FSH response to TRH–Gn RH in different types of hypogonadism and in controls (mean ± S.E.M.)*

| | FSH, U/l | | | | | | ΔFSH |
|---|---|---|---|---|---|---|---|
| | 0 min | 15 min | 30 min | 60 min | 120 min | 180 min | |
| Controls (n=8) | 7.31 ±0.94 | 9.93 ±1.53 | 12.36* ±1.71 | 13.95* ±2.41 | 11.00 ±2.17 | 10.62 ±3.00 | 6.99* ±1.47 |
| Primary testicular failure with normal basal gonadotropin level (n=6) | 5.51 ±1.28 | 7.82 ±1.76 | 8.15 ±1.93 | 9.73 ±2.22 | 7.88 ±1.35 | 7.50 ±1.70 | 4.50* ±1.40 |
| Primary testicular failure with elevated basal gonadotropin level (n=7) | 27.71** ±2.52 | 42.50*, ** ±4.24 | 43.00*, ** ±4.33 | 46.71*, ** ±4.97 | 39.29*, ** ±8.23 | 40.00*, ** ±3.58 | 21.85*, ** ±3.43 |
| Klinefelter's syndrome (n=9) | 30.42** ±5.76 | 41.80** ±6.68 | 41.52** ±6.02 | 40.70** ±6.38 | 40.85** ±5.62 | 33.40** ±4.57 | 16.98*, ** ±3.57 |
| Hypogonadotrophic hypogonadism (n=5) | 0.68** ±0.28 | 1.68* ±0.70 | 1.62* ±0.81 | 1.96* ±0.86 | 1.80* ±0.76 | — | 1.44*, ** ±0.55 |

n = number of patients
* Compared to basal level (time 0) the differences are significant statistically
** Compared to the controls the differences are significant statistically

**Table V**

*LH responses to TRH–Gn RH in different types of hypogonadism and in controls (mean ± S.E.M.)*

| | LH, U/l | | | | | | ΔLH |
|---|---|---|---|---|---|---|---|
| | 0 min | 15 min | 30 min | 60 min | 120 min | 180 min | |
| Controls (n=8) | 11.65 ±1.35 | 48.88* ±10.79 | 67.13* ±11.18 | 47.56* ±8.59 | 31.13* ±4.51 | 17.22* ±2.24 | 55.47* ±10.53 |
| Primary testicular failure with normal basal gonadotropin level (n=6) | 11.38 ±1.60 | 37.11* ±6.59 | 47.40* ±11.80 | 32.60* ±4.16 | 20.66* ±2.35 | 16.80* ±1.07 | 40.39* ±8.74 |
| Primary testicular failure with elevated basal gonadotropin level (n=7) | 20.00* ±1.86 | 97.63*, ** ±11.77 | 102.29* ±17.60 | 85.13*, ** ±7.04 | 63.29*, ** ±6.60 | 52.12*, ** ±8.21 | 95.85*, ** ±9.50 |
| Klinefelter's syndrome (n=9) | 29.97** ±4.09 | 99.75*, ** ±9.37 | 120.78*, ** ±16.52 | 107.88*, ** ±13.04 | 78.43*, ** ±8.36 | 72.80*, ** ±7.58 | 92.37* ±14.87 |
| Hypogonadotrophic hypogonadism (n=5) | 3.68** ±2.06 | 7.66** ±4.40 | 10.62** ±6.23 | 9.12** ±5.57 | 6.52** ±4.00 | — | 6.88** ±4.59 |

n = number of patients
* Compared to basal level (time 0) the differences are significant statistically
** Compared to the controls the differences are significant statistically

increase in TSH, PRL and hGH independently from the kariotype, except in hypogonadotrophic hypogonadism where the PRL increase was slighter. The testosterone levels did not influence the elevation of the hormone levels. The peak and the duration of FSH and LH differed after TRH–GnRH treatment, depending on the basal level of these hormones.

The PRL peak was found to be attenuated in male hypogonadotrophic hypogonadism [25] and exaggerated in primary testicular failure. Others [1] found a normal response while Yamaji et al. [31] an attenuated result in 4 out of their 8 patients. Our patients with hypogonadotrophic hypogonadism showed a significantly lesser increase in PRL than did the controls in 15 minutes. If all our 22 patients with primary testicular failure were compared to the hypogonadotrophic hypogonadism group, a significant difference was found in PRL. This is thought to be due to the lesser amount or rather the decreased activity of the pituitary PRL cells. Repeated TRH loads would be probably more effective. The testosterone level did not influence this reaction since in the Klinefelter's syndrome suffering patients with a low or normal testosterone level the response was normal. Testosterone treatment did not modify the attenuated PRL reaction of the patients of Yamaji et al. [31] and did not influence the reaction of PRL after TRH bolus in healthy volunteers [16] or in patients with hypogonadism [21]. In males with azoospermia an attenuated PRL response was found by Goldhaber et al. [11] and exactly the opposite, an exaggerated one, by Dickerman et al. [8] while a normal one by others [5, 13] and ourselves. In female patients with hypogonadotrophic hypogonadism a lower basal level but a normal increase after TRH was observed [11, 13, 22, 25].

During clomifene treatment which augmented the FSH, LH levels, a decrease in the serum PRL level was seen by Djordjevic [9]. This might have been due to the clomifene treatment itself and not to the elevated FSH and LH levels since in our patients and controls with elevated or normal FSH and LH, the increase in PRL was similar after TRH loading. The same normal increase was found in our female patients with streak gonad syndrome and elevated gonadotropin level [3].

The hGH levels did not differ between patients and controls and their reaction to TRH–GnRH loading was also similar. Metoclopramide was found to increase the hGH level in male hypogonadism but not in controls [6]. Similarly, GnRH was found to increase the hGH level in nine of 16 and TRH in one of 14 Klinefelter's syndrome patients [8]. In Turner's syndrom a low serum hGH level [10] and altered response to glucose [20] and insulin [18] was found. Therapy with small amounts of oestrogen was able to normalize the latter response [18]. A protracted and exaggerated response of FSH and LH was found after GnRH in patients with elevated basal FSH and LH levels, and an attenuated one with decreased basal levels. This could be due to

the gonadotropic cell activity of the hypophysis. Active or superactive cells were found to react more easily to the releasing hormone than do inactive cells, as observed also by Spitz et al. [27]. An attenuated reaction of FSH and LH to GnRH was found in female hypogonadotrophic hypogonadism [22] as well as in prepuberal boys [2, 7]. In female hypogonadotrophic hypogonadism the FSH and LH release was normalized after prolonged gonadotropic releasing hormone treatment [22].

The testosterone levels per se did not influence the reaction of FSH and LH after TRH–GnRH only through a feed-back mechanism as in our patients with hypogonadotrophic hypogonadism and Klinefelter's syndrome with similarly low testosterone levels a completely different type of reaction could be seen. Similarly, no correlation was found between testosterone level and gonadotropin release in females [30].

The oestrogen level is known to influence gonadotropin release in both females [14] and males [17] but this was not measured in our patients. According to our results the trop hormones and their reactions are unchanged in primary testicular failure except for the gonadotropins, while in hypogonadotrophic hypogonadism PRL shows a decreased, reactivity.

## Acknowledgements

We are indebted to J. Borvendég M. D., Institute for Drug Research, Budapest, for the generous donation of GnRH.

## REFERENCES

1. Antaki, A., Somma, M., Wyman, H., van Campenhaut, J.: Hypothalamic-pituitary function in the olfacto-genital syndrome. J. Clin. Endocr. **38,** 1083–1089 (1974)
2. Beatriz, M., De Behar, R., Mendilaharzu, H., Rivarola, M., Heinrich, H. H., Begada, C.: Gonadotropin response to LH–RH in patients with hypogonadotrophic hypogonadism. Helv. paediat. Acta **32,** 227–235 (1977)
3. Bősze, P., Kovács, Zs., Egyed, J., László, J., Szilágyi, G.: Thyrotrophin and prolactin responses to thyrotrophin-releasing hormone in patients with streak gonad syndrome. Acta endocr. (Kbh.) **102,** 179–184 (1983)
4. Burman, K. D., Diamond, R. C., Noel, G. L., Earll, J. M., Frantz, A. G., Wartofsky, I.: Klinefelter's syndrome examination of thyroid function and the TSH and PRL responses to thyrotropin-releasing hormone prior to and after testosterone administration. J. clin. Endocr. **41,** 1161–1166 (1975)
5. Cheikh, I., Hamilton, B. P., Hsu, T. H., Wiswell, J. G.: Response of TSH and prolactin to TRH in Klinefelter's syndrome. Endocrinology Suppl. **96,** A206 (1975)
6. Cohen, N. H., Hay, D. I., Beastall, H. G., Thomson, A. J.: Metoclopramide induced growth hormone release in hypogonadal males. Clin. Endocr. **11,** 95–97 (1979)
7. De Lange, E. W., Snoep, C. M., Doorenbos, H.: The effect of LH–RH infusion on serum LH, FSH and Testosterone in boys with advanced puberty, delayed puberty and hypogonadotrophic hypogonadism. Acta endocr. (Kbh.) **89,** 209–216 (1978)
8. Dickerman, Z., Rachmel, A., Gil-Ad, I., Prager-Lewin, R., Galatzer, A., Laron, Z.: Rise in plasma growth hormone in response to exogenous LRH in Klinefelter's syndrome. Clin. Endocr. **15,** 403–410 (1981)
9. Djordjevic, B. P., Djuric, S. D., Micic, D. D.: Gonadotropins, testosterone and prolactin values in serum of sterile males before and after treatment with clomiphen. Acta endocr. (Kbh.) Suppl. **225,** 149 (1979)

10. Duke, E. M. C., Hussein, D. M., Hamilton, W.: Turner's syndrome associated with growth hormone deficiency. Scot. med. J. **26,** 240–244 (1981)
11. Goldhaber, G., Zuckerman, Z., Shani, J., Cohen, J. J.: Effect of thyrotropin-releasing hormone on serum prolactin levels in man with azoospermia. J. Reprod. Fertil. **49,** 135–137 (1977)
12. Göretzlehner, G., Rudolf, K., Kunkel, S.: Combination test with arginine, GnRH, and TRH in adolescent patients with gonadal dysgenesis before and after treatment with sexual steroids. Acta endocr. (Kbh.) Suppl. **225,** 391 (1979)
13. Hagen, C., McNeilly, S. A., Arroe, M., Emmertsen, K., Froland, A.: Prolactin levels in gynaecomastia related to Klinefelter's syndrome. Lancet **2,** 57 (1974)
14. Hoff, D. J., Lasley, L. B., Wang, F. C., Yen, C. S. S.: The two pools of pituitary gonadotropin: regulation during menstrual cycle. J. clin. Endocr. **44,** 302–312 (1977)
15. Hungerford, D. A.: Leukocytes cultured from small inocula of whole blood and the preparation of metaphase chromosomes by treatment with hypotonic KCl. Stain Technol. **40,** 333–338 (1965)
16. Kicovic, P. M., Luisi, M., Franchi, F., Krempl, S.: Circulating prolactin and its response to TRH following administration of testosterone undecanoate in normal men. J. endocr. Invest. **1,** 359–363 (1978)
17. Kulin, H. F., Reiter, O. E.: Gonadotropin and testosterone measurements after estrogen administration to adult men, prepubertal and pubertal boys and men with hypogonadotropism: Evidence for maturation of positive feed-back in the male. Pediat. Res. **10,** 46–51 (1976)
18. Laczi, F., Julesz, J., Janáky, T., László, F. A.: Growth hormone reserve capacity in Turner's syndrome. Horm. Metab. Res. **11,** 664–666 (1979)
19. LeRoith, D., Liel, Y., Sack, J., Livshin, Y., Laufer, N., Schenker, J., Spitz, M. I.: The TSH response to TRH is exaggerated in primary testicular failure and normal in the male castrate. Acta endocr. (Kbh.) **97,** 103–108 (1981)
20. Lindsten, J., Cerasi, E., Luft, R., Ultquist, G.: The occurrance of abnormal insulin and growth (hGH) responses to sustained hyperglycaemia in a disease with sex chromosome aberrations (Turner's syndrome). Acta endocr. (Kbh.) **56,** 107–131 (1967)
21. Morley, E. J., Sawin, T. C., Carlson, E. H., Longcope, Ch., Hershman, M. J.: The relationship of androgen to the thyrotropin and prolactin responses to thyrotropin-releasing hormone in hypogonadal and normal man. J. clin. Endocr. **52,** 173–176 (1981)
22. Rabinowitz, D., Spitz, M. I., Benveniste, R.: Isolated gonadotropin deficiency. In: Givens, R. J. (ed.): Endocrine Causes of Menstrual Disorders. Year Book Med. Publ. Inc., Chicago 1978, pp. 181–190
23. Samaan, N. A., Stepanas, A. V., Danziger, J., Trujillo, J.: Reactive pituitary abnormalities in patients with Klinefelter's and Turner's syndromes. Arch. intern. Med. **139,** 198–201 (1979)
24. Smals, A. G. H., Kloppenborg, P. W. C., Lequin, R. L., Bees, L., Ross, A., Benraad, T. J.: The pituitary-thyroid axis in Klinefelter's syndrome. Acta endocr. (Kbh.) **84,** 72–79 (1977)
25. Spitz, M. I., Almaliach, U., Rosen, E., Polishuk, W., Rabinowitz, D.: Dissociation of prolactin responsiveness to TRH and chlorpromazine in women with isolated gonadotropin deficiency. J. clin. Endocr. **45,** 1173–1178 (1977)
26. Spitz, M. I., Zylber, E., Cohen, H., Almaliach, U., LeRoith, D.: Impaired prolactin response to Thyrotropin-Releasing Hormone in isolated gonadotropin deficiency and exaggerated response in primary testicular failure. J. clin. Endocr. **48,** 941–945 (1979)
27. Spitz, M. I., Halperin, Y., Shilo, Sh., LeRoith, D., Zylber-Haran, E., Livshin, Y., Laufer, N., Schenker, J.: Clomiphene attenuates the exaggerated prolactin response to thyrotropin-releasing hormone and metoclopramide occurring in primary testicular failure. J. clin. Endocr. **52,** 289–293 (1981)
28. Szilágyi, G., Bősze, P., Egyed, J., Kovács, Zs., László, J.: Effect of TRH in the serum HGH level in patients with gonadal dysgenesis. Acta med. Acad. Sci. hung. **36,** 270–271 (1979)
29. Vermeulen, A., Verdonck, L.: Testosterone assays by competitive protein binding. In: Diczfalusy, E. (ed.): Steroid Assay by Protein Binding. Karolinska Symposia on Research Methods in Reproductive Endocrinology, Bogtrykkeriet Forum, Stockholm 1970, pp. 239–256
30. Vierhapper, H., Waldhausl, W., Nowotny, P.: Gonadotrophin release upon intravenous administration of a long-acting analogue luteinizing hormone-releasing hormone in female with increased plasma-androgens. Acta endocr. (Kbh.) **91,** 577–590 (1979)
31. Yamaji, T., Shimamoto, K., Kosaka, K., Isurugi, K.: Heterogenity of prolactin and TSH response to TRH in hypogonadotropic hypogonadism. J. clin. Endocr. **45,** 319–323 (1977)

*Acta Medica Hungarica 41 (4) pp. 185–193 (1984)*

# HYPERPROLACTINAEMIA AND FEMALE INFERTILITY

G. Godó

DEPARTMENT OF OBSTETRICS AND GYNAECOLOGY, UNIVERSITY MEDICAL SCHOOL, SZEGED, HUNGARY

(Received July 19, 1983)

The clinical use of bromocriptine was investigated in 50 hyper- and 30 normoprolactinaemic women attending an infertility clinic and presenting with anovulatory cycles, oligomenorrhoea or amenorrhoea and the complaint that they had failed to become pregnant. The results confirmed that bromocriptine is effective in the treatment of hyperprolactinaemic states. Bromocriptine supresses prolactin secretion irrespective of the underlying pathologic process.

Hyperprolactinaemia in humans is frequently associated with anovulation. Serum prolactin values showed no close correlation with the degree of menstrual abnormalities or galactorrhoea. Basal FSH and LH levels and the gonadotropin response to LH–RH were essentially normal in hyperprolactinaemia. Circulating $E_2$ levels were largely subnormal suggesting an inhibitory effect of prolactin on ovarian $E_2$ production. Prolactin levels over 100 ng/ml are suggestive of pituitary adenoma.

**Keywords:** female infertility, prolactin, bromocriptine.

## Introduction

During the past decade a large number of papers have dealt with prolactin but our knowledge concerning the physiological importance of the hormone is still limited. Introduction into clinical medicine of prolactin radioimmunoassay, increasing sophistications in the roentgenographic diagnosis of pituitary microadenomas, the introduction of transsphenoidal pituitary microsurgery, and the discovery of drugs that selectively stimulate dopaminergic receptors and inhibit prolactin secretion, have given insight into the neuroendocrine control of prolactin secretion and revolutionized the therapy of the galactorrhoea-amenorrhoea syndromes [10, 11, 19, 21].

The syndromes of galactorrhoea and amenorrhoea were recognized long before the presence of prolactin in the human organism had been established.

Send offprint requests to G. Godó, Department of Obstetrics and Gynaecology, H-6725 Szeged, Semmelweis u. 1, Hungary

Although the primary cause was unknown, the reports of patients suffering from the syndromes pointed to a common denominator [22]. Careful examination of the sella turcica by tomography revealed minute signs of pituitary enlargement in a great number of cases [21]. It has now become clear that the common denominator in these syndromes (Chiari–Frommel, Argonz-del Castillo, Forbes–Albright) consists of elevated plasma levels of prolactin [15]. Hyperprolactinaemia was originally diagnosed if the prolactin level exceeded 25 ng/ml plasma.

Bromocriptine (Parlodel®, Sandoz, Basel), a dopamine receptor agonist, is a potent inhibitor of prolactin secretion [15, 17, 18]. Lowering of plasma prolactin either surgically or medically promptly restores ovarian function which indicates that it is the prolactin itself which through some as yet unexplained feedback mechanism controls hypothalamic Gn–RH in hyperprolactinaemic women. There is, however, a considerable amount of evidence that excess prolactin may also act directly on the ovaries, either by interference with the effect of FSH or by interfering with the gonadotropin-mediated steroidogenesis. This results in inadequate feedback mechanisms both at the hypothalamic and pituitary levels and this may at least in part explain the hypogonadism [5, 8, 13, 16].

The aim of the present paper is to review and discuss data regarding the influence of hyperprolactinaemia on gonadal function in the human female.

## Material and methods

In 50 hyperprolactinaemic and 30 normoprolactinaemic patients aged between 18 and 40 years with normal sella roentgenograms and visual fields, informed consent was obtained for the use of bromocriptine (Parlodel®, Sandoz). They were attending the Endocrine Outpatient Clinic at our Department. They received 2.5 mg bromocriptine b.i.d. In all patients basal body temperature was monitored and plasma progesterone was measured by radioimmunoassay. They received the drug until galactorrhoea ceased and menses had resumed or pregnancy occurred, or until side-effects led to discontinuation of therapy. The treatment was

**Table I**

*Results of patients treated for functional infertility*

| Treatment with | No. treated | Ovulated % | Conceived % |
|---|---|---|---|
| Bromocriptine | | | |
| 1. normal plasma prolactin | 30 | 10–33 | 6–20 |
| 2. elevated plasma prolactin | 50 | 39–78 | 28–56 |
| Clomiphene citrate (normal plasma prolactin) | 50 | 27–54 | 18–36 |
| Spontaneous | 30 | 6–20 | 4–13 |

discontinued as soon as a positive pregnancy test was obtained. Serum prolactin levels were measured weekly. Circulating prolactin levels over 100 ng/ml were strongly suggestive of an organic pituitary lesion. Lower prolactin values did not, however, exclude this possibility. Normoprolactinaemic anovulatory patients treated with clomiphene citrate and such patients without any drug served as control groups (Table I).

Twenty-five patients who initially presented with galactorrhoea-amenorrhoea, fell into five diagnostic groups (Fig. 5). All patients were evaluated by history, physical examination, plasma prolactin, FSH, LH and oestradiol levels and by lateral and postero-anterior skull films, biplanar tomography of the sella and visual-field testing. Patients with regular menses served as control subjects for endocrine studies. The pituitary gonadotrophic function was also studied: luteinizing hormone (LH) and follicle-stimulating hormone (FSH) were measured in the basal state and at 15, 30, 60, 90, and 120 min after administration of LH–RH (100 $\mu$g intravenously).

Plasma hormones were measured by radioimmunoassay in the morning basal fasting state [11]. Results are expressed as means $\pm$S.E.M.

## Results

In normal subjects studied in the early follicular phase, the response of serum LH to LH–RH varied over a wide range (20–60 mU/ml), with peak values reached at 15 min in three patients and 30 min in two patients. The FSH increase ranged between 15 and 30 mU/ml, with peak levels occurring at 60 min in three patients and at 90 min in two patients. The mean increase in LH and FSH did not differ significantly between patients with amenorrhoea-galactorrhoea and normal control subjects (Fig. 1).

The plasma prolactin levels of the 20 anovulatory women are shown in Fig. 2 as compared to values found in patients with amenorrhoea-galactorrhoea. The mean prolactin level in this group was higher (24 ng/ml) than the mean value observed in normal women (14 ng/ml), but lower than that found in patients with amenorrhoea-galactorrhoea syndrome (63 ng/ml).

Circulating FSH-levels in hyperprolactinaemic patients were similar to those found during the follicular phase of the normal menstrual cycle in healthy women, except for 5 patients with borderline low values. Plasma LH

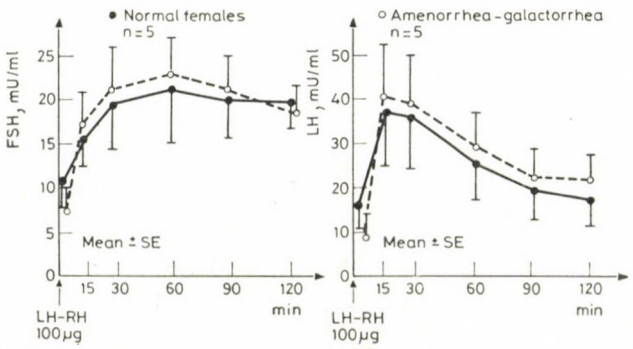



*Fig. 1.* Mean $\pm$ S.E.M. plasma FSH and LH levels and the increment after intravenous 100 $\mu$g LH–RH in normal women studied in the early follicular phase of the cycle, and patients with amenorrhoea-galactorrhoea

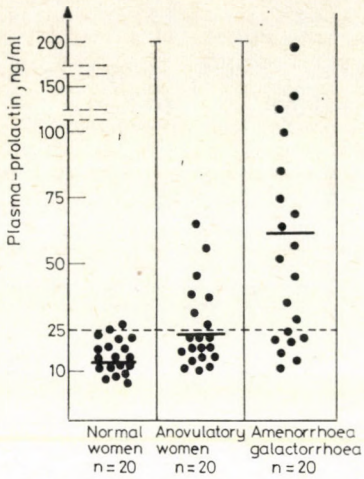



*Fig. 2.* Mean prolactin concentrations were higher in infertile patients, but overlapped prolactin concentrations in normally menstruating women. The patients with amenorrhoea-galactorrhoea had the highest prolactin levels

concentrations were also comparable to normal follicular values, except in 7 patients with borderline low and another 3 with high levels. The overall mean FSH and LH values of patients with hyperprolactinaemic infertility were in the lower areas of the normal follicular range (Fig. 3).

Plasma oestradiol levels in hyperprolactinaemic patients were significantly lower than those seen during the mid-late follicular phase of the ovulatory cycle in normal women (Fig. 4). Several patients, however, showed high $E_2$ values and signs of hypoestrogenism such as breast and vaginal skin hypotrophy were not found in any patient. In addition, there was a small but significant inverse correlation between plasma $E_2$ and prolactin levels ($P < 0.05$).

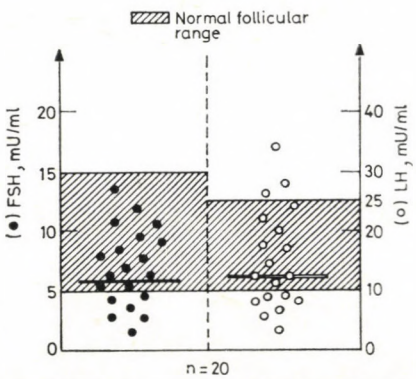



*Fig. 3.* Basal plasma FSH and LH levels in hyperprolactinaemic patients. The shaded areas represent the range of follicular phase values in ovulatory cycles of healthy women. The horizontal lines indicate mean values

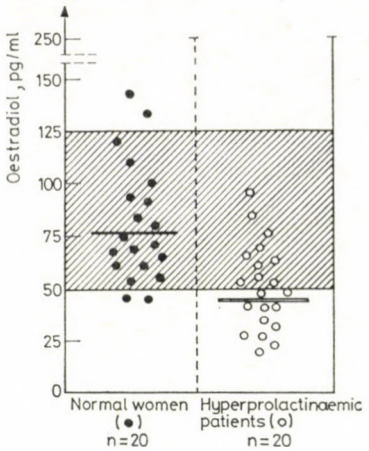



*Fig. 4.* Basal plasma levels of oestradiol-17-beta in normally menstruating women and in hyperprolactinaemic patients. The shaded area represents the range of mid-late follicular phase of the ovulatory cycle in normal women. The horizontal lines indicate mean values

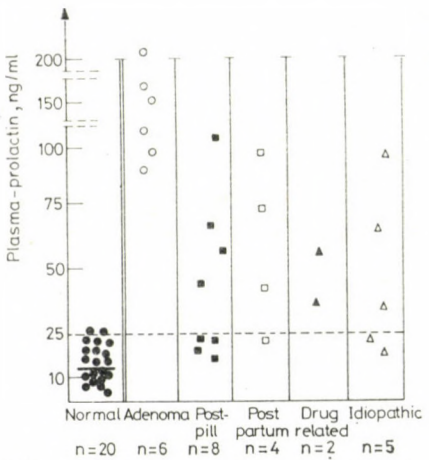



*Fig. 5.* Mean prolactin levels in normal women and in patients with galactorrhoea-amenorrhoea secondary to prolactin-secreting adenomas and post-pill, post-partum, drug-related, and idiopathic galactorrhoea-amenorrhoea

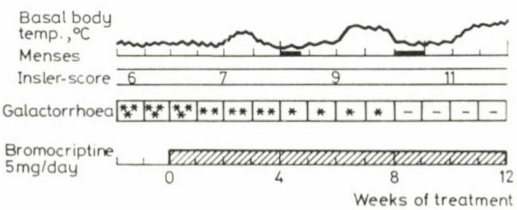



*Fig. 6.* Clinical findings during bromocriptine therapy in a woman suffering from galactorrhoea and amenorrhoea who conceived during the 3rd treatment cycle. The Insler-score is a method for estimating the physical qualities of the cervical mucus

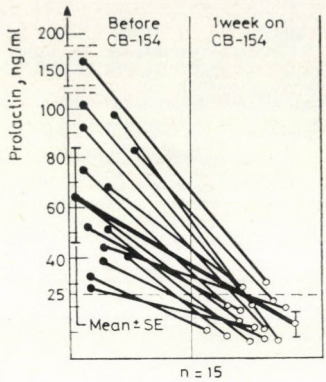



*Fig. 7.* Changes in circulating prolactin levels in a group of patients with galactorrhoea-amenorrhoea syndrome after one week of bromocriptine (CB-154, Sandoz) treatment

Basal prolactin levels (Fig. 5) in 20 normal women ranged from 4 to 28 ng/ml (mean 13.8 ng/ml). The upper limit of normal range in our laboratory was taken as 25 ng/ml. In patients with adenoma, basal prolactin levels were greater than 100 ng/ml in five of six cases, one patient with an adenoma had a basal prolactin of only 89 ng/ml. Prolactin levels in four of the eight patients with post-pill galactorrhoea-amenorrhoea were normal and elevated in the other four patients. One patient without adenoma had a basal prolactin above 100 ng/ml. In patients with post-partum galactorrhoea-amenorrhoea, prolactin levels ranged from normal to 97 ng/ml. Basal levels in the two drug-related diseases were moderately elevated, and were quite variable in the patients with "idiopathic" galactorrhoea-amenorrhoea ranging from normal values to 90 ng/ml.

The clinical findings during initiation of treatment in one patient are shown in Fig. 6. Within 8 weeks of therapy her galactorrhoea had disappeared completely and examination of the uterine cervix revealed rich mucus showing signs of oestrogenic activity. Her body temperature curve shifted at this time and 2 weeks later she menstruated for one week. During the next 4 weeks she had a normal menstrual cycle with a biphasic basal temperature curve. Fourteen weeks after starting the therapy, the pregnancy test showed HCG activity as in pregnant women.

During bromocriptine treatment irrespective of wheather the prolactin levels were greatly or only slightly elevated, there was a dramatic fall in the circulating prolactin level (Fig. 7).

The results of bromocriptine treatment are shown in Table I. Eighty patients were treated with bromocriptine. Thirty-nine of the fifty with hyperprolactinaemia ovulated and 28 conceived. In the group of patients with normal plasma prolactin levels only 33% ovulated and 20% conceived.

## Discussion

The mechanism by which hyperprolactinaemia interferes with pituitary gonadotropin secretin and ovulation, remains to be elucidated. A different effect of high levels of prolactin on the hypothalamus and/or the pituitary has been suggested, as well as a blocking effect of prolactin at the ovarian level [20]. These hypotheses are supported by studies showing that suppression of elevated prolactin levels with bromocriptine in women with amenorrhoea, with or without galactorrhoea, results in resumption of cyclic gonadotropin secretion, ovulation, and fertility [2, 3, 14]. On the basis of previous studies, it is suggested that infertile women with hyperprolactinaemia could benefit from the administration of a prolactin suppressor in order to achieve cyclic ovulation and pregnancy.

Patients requiring ovulation induction should be treated with clomiphene citrate if their plasma FSH, LH and prolactin levels are normal, but they need bromocriptine when the plasma prolactin is elevated and the presence of a pituitary tumour has been excluded [4, 9]. The use of bromocriptine with a view to causing a reduction in size of prolactinomas and/or preventing further growth is currently under investigation and appears promising [1]. Radiotherapy provides a further method of treatment.

Orally administered bromocriptine is rapidly and completely absorbed. Because of its tendency to cause like L-dopa nausea, vomiting and postural giddiness in the early days of therapy, dosage should begin with 1.25 mg twice daily, commencing with an evening dose taken during a meal. After some days, such symptoms have usually subsided and the conventional dose of 2.5 mg two or three times daily with a meal can be given. There is currently no suggestion that the drug has teratogenic properties in humans.

If hyperprolactinaemia is demonstrated, its cause must first be established. Provided that hypothyroidism, drug ingestion (phenothiazines, butyrophenones, alpha-methyldopa, reserpine, oestrogen), etc., have been eliminated, attention must be directed to pituitary radiology, proceeding to polytomography [12]. The existence of a symptomatic tumour is generally accepted as an indication for surgical intervention, although there are several recent reports where patients with prolactinoma were treated with bromocriptine.

The major practical difficulty in patients presenting with amenorrhoea-galactorrhoea is to establish whether the diagnosis is that of pituitary adenoma, and to assign a cause if pituitary radiology is normal and no other lesion is evident, i.e. idiopathic hyperprolactinaemia. Whether idiopathic hyperprolactinaemia results from a functional disturbance of inhibitory prolactin control, excessive secretion of a hypothalamic releasing factor, or from a clinically undetectable microadenoma is not known.

Franks et al. [7] found that, although plasma oestradiol levels were often in the menopausal range, basal plasma gonadotrophin concentrations were generally within or just above the normal range for the follicular phase and, therefore, had not risen as high as expected for the marked degree of oestrogen deficiency. In addition to the postulated effect on the ovary, hyperprolactinaemia probably also inhibits gonadotrophin secretion. Presumably, release more than synthesis is impaired, since such patients typically show normal or exaggerated responses of LH and FSH to injections of LHRH, despite normal basal gonadotrophin concentrations.

## REFERENCES


1. Archer, D. F., Lattanzi, D. R., Moore, E. E., Harger, J. H., Herbert, D. L.: Bromocriptine treatment of women with suspected pituitary prolactin-secreting microadenomas. Am. J. Obstet. Gynecol. **143,** 620–625 (1982)
2. Bergh, T., Nillius, S. J., Wide, L.: Bromocriptine treatment of 42 hyperprolactinaemic women with secondary amenorrhoea. Acta Endocrinol. (Copenh.) **88,** 435–451 (1978)
3. Bohnet, H. G., Dahlen, H. G., Wuttke, W., Schneider, H. P. G.: Hyperprolactinemic anovulatory syndrome. J. Clin. Endocrinol. Metab. **42,** 132–143 (1976)
4. Cooke, I. D.: The role of bromocriptine in the management of infertility. Res. Clin. Forums **3,** No. 1, 17–28 (1981)
5. Corbey, R. S., Lequin, R. M., Rolland, R.: Hyperprolactinemia and secondary amenorrhea. In Crosignani, P. G., Robyn, C. (eds): Prolactin and Human Reproduction. Academic Press, London 1977, pp. 203–215.
6. Franks, S., Murray, M. A. F., Jequier, A. M., Steele, S. J., Nabarro, J. D. N. and Jacobs, H. S.: Incidence and significance of hyperprolactinaemia in women with amenorrhoea. Clin. Endocrinol. (Oxf.) **4,** 597–607 (1975)
7. Franks, S., Murray, M. A. F., Steele, S. J., Nabarro, J. D. N. and Jacobs, H. S.: Hyperprolactinaemic amenorrhoea: clinical and endocrine features. Acta Endocrinol. (Copenh.) Suppl. **199,** 204 (1975)
8. Glass, M. R., Shaw, R. W., Butt, W. R., Logan Edwards, R., London, D. R.: An abnormality of oestrogen feed-back in amenorrhoea-galactorrhoea. Br. Med. J. **3,** 274–275 (1975)
9. Godó, G., Sas, M.: Die klinische Bedeutung der antiprolaktinämischen Behandlung bei der funktionellen Infertilität. Zbl. Gynäkol. **101,** 1187–1194 (1979)
10. Hardy, J.: Transsphenoidal microsurgery of the normal and pathological pituitary. Clin. Neurosurg. **16,** 185–217 (1969)
11. Hwang, P., Guyda, H., Friesen, H.: A radioimmunoassay for human prolactin. Proc. Natl. Acad. Sci. USA **68,** 1902–1906 (1971)
12. Marrs R. P., Kletzky, O. A., Teal, J., Davajan, V., March, C., Mishell, D. R.: Comparison of serum prolactin, plain radiography and hypocycloidal tomography of the sella turcica in patients with galactorrhea. Am. J. Obstet. Gynecol. **135,** 467–469 (1979)
13. McNatty, K. P., Sawers, R. S., McNeilly, A. S.: A possible role for prolactin in control of steroid secretion by the human Graafian follicle. Nature **250,** 653–655 (1974)
14. Del Pozo, E., Varga, L., Wyss, H., Tolis, G., Friesen, H., Wenner, R., Vetter, L., Uettwiler, A.: Clinical and hormonal response to bromergocryptine (CB-154) in the galactorrhea syndromes. J. Clin. Endocrinol. Metab. **39,** 18–26 (1974)
15. Rolland, R., Schellekens, L. A., Lequin, R. M.: Successful treatment of galactorrhoea and amenorrhoea with subsequent restoration of ovarian function by a new ergot alkaloid 2-brom-alpha-ergocryptine. Clin. Endocrinol. (Oxf.) **3,** 155–166 (1974)
16. Saito, T., Saxena, B. B.: Specific receptors for prolactin in the ovary. Acta Endocrinol. (Copenh.) **80,** 126–137 (1975)
17. Sas, M., Godó, G., Morvay, J.: Behandlung hyperprolactinämischer Zustände mit Brom-Ergokryptin. Zbl. Gynäkol. **100,** 1501–1507 (1978)
18. Thorner, M. O., Besser, G. M.: Hyperprolactinaemia and gonadal function: results of bromocriptine treatment. In: Crosignani, P. G., Robyn, C. (eds). Prolactin and Human Reproduction. Academic Press, London 1977, pp. 285–301

19. Thorner, M. O., McNeilly, A. S., Hagan, C. et al.: Long-term treatment of galactorrhoea and hypogonadism with bromergocriptine. Br. Med. J. **2,** 419–422 (1974)
20. Tyson, J. E., Khojandi, M., Huth, J., Smith, B., Thomas, P.: Inhibition of cyclic gonadotropin secretion by endogenous human prolactin. Am. J. Obstet. Gynecol. **121,** 375–379 (1975)
21. Vezina, J. L., Sutton, T. J.: Prolactin-secreting pituitary microadenomas: roentgenologic diagnosis. Am. J. Roentgenol. **120,** 46–54 (1974)
22. Young, R. L., Bradley, E. M., Goldzieher, J. W., Myers, P. W., Lecocq, F. R.: Spectrum of nonpuerperal galactorrhea: report of two cases evolving through the various syndromes. J. Clin. Endocrinol. **27,** 461–466 (1967)

*Acta Medica Hungarica, 41 (4), pp. 195—202 (1984)*

# EPISODIC SECRETION OF HORMONES AND THE DIAGNOSTIC VALUE OF SINGLE BLOOD ESTIMATES I. LH, FSH, PROLACTIN*

G. Siklósi, L. Bakos, S. Csömör jr., Z. Marcsek, F. Olajos

SECOND DEPARTMENT OF OBSTETRICS AND GYNAECOLOGY SEMMELWEIS UNIVERSITY MEDICAL SCHOOL, BUDAPEST, HUNGARY

(Received June 9, 1983)

The episode fluctuation of serum LH, FSH and prolactin levels in peripheral blood was studied to determine the reliability of single estimates of the average blood level. Radioimmunoassay of the hormones was performed in serum withdrawn every 10 min between 8 and 12 h a.m. from 8 healthy women in various periods of the menstrual cycle. The rate of fluctuation of the hormone levels was characterized by the within-person coefficient of variation of single estimates, which averaged 30.3, 15.1 and 19.2% for LH, FSH and prolactin, respectively. Due to a pronounced fluctuation of gonadotropin levels, analysis of several serum samples is recommended to approach the actual mean hormone level.

**Keywords:** Secretion of hormones, LH, FSH, prolactin.

## Introduction

Recent studies have demonstrated an episodic secretion of many hormones and an oscillation pattern of their daily serum level. The bioavailability and the rate of secretion of hormones is routinely approached in clinical practice by their blood level. The greater the fluctuation of hormone concentration in peripheral circulation, the less reliable is a single estimate in representing the average daily hormone concentration.

In the present paper the episodic fluctuation of the blood level of gonadotropins was studied to assess the reliability of a single estimate for clinical purposes.

## Patients and methods

Eight apparently healthy women aged 23 to 35 years volunteered for this study. They had regular menstrual cycle lasting for 26 to 30 days. Their weight was ideal with a deviation of at most $\pm 10\%$. Onset of ovulation and physiological corpus luteum function were controlled by measurement of basal body temperature and serial determination of the serum progesterone

Send offprint requests to G. Siklósi, H-1082 Budapest, Üllői út 78/a, Hungary

* Presented in part at the 9th Congress of the Hungarian Society of Endocrinology and Metabolism, Szeged, Hungary 1979.

level. The episodic fluctuation of blood hormone concentrations was studied 90 min or more after awakening. Blood samples were withdrawn after inserting a cannula into the cubital vein every 10 min between 8 an 12 h a.m. The 25 blood samples obtained were immediately centrifuged and stored at −20 °C until processing. To exclude interassay variation of methodological errors, all samples of a subject were worked up within one assay. The mean value of the 25 estimates was considered, to indicate the mean blood hormone level. Deviation of single estimates from the mean was characterized by the coefficient of variation of 25 measurements, and the maximum deviation of individual values was assessed at 95% confidential limits (2 S.D.). To compare inter-person estimates, the values were expressed in percentage increments from the intra-person average value.

## Hormone determinations

### *LH radioimmunoassay*

LH determination in serum was performed by homologous, double antibody radioimmunoassay according to Midgley [7] with the modifications proposed by Reuter et al. [15]. The LH antiserum used was a specific anti-human rabbit serum (Calbiochem). A 125 I-labelled and purified human LH tracer (CIS Cea-Ire-Sorin LH-125-B) was further purified on 10×100 mm Sephadex G-100 (Pharmacia) column 24 h before use [11]. An LH standard preparation of the "1st International Reference Preparation of Human Pituitary Gonadotropins FSH and LH 69/104 (WHO)" with a biological activity of 1 IU FSH = 0.167 mg and 1 IU LH = 0.668 mg was employed [3]. The normal rabbit serum and bovine serum albumin, and as second antibody, goat antiserum to rabbit gamma globulin were purchased from Calbiochem. Phosphate buffer (0.01 mol pH 7.6 including 0.14 mol NaCl, 0.05 mol EDTA–$Na_2$, 0.05% Na azide, 1.0% BSA) was used. The first antiserum solution in the buffer contained 0.5% normal rabbit serum.

The radioassay was performed in glass tubes in an incubation volume 700 μl. The working standards were 1.25–2.5–5.0–10.0 and 20.0 mIU/ml of reference LH. All dilutions were made by phosphate buffer indicated above. To duplicates of 200 μl serum and standard solutions, 10 000 dpm tracer in 100 μl buffer and 200 μl antiserum were added, vortexed and incubated at room temperature for 16 to 24 h. The second antibody in 200 μl was then added, vortexed and incubated at 4 °C for 16 to 24 h. Thereafter, the samples were centrifuged at 4 °C the supernatan was decanted and radioactivity measured in a GAMMA NK 350a counter (Gamma Works, Budapest). Results were calculated by a logit-log transformation of measurements [16].

### *FSH radioimmunoassay*

Serum FSH was determined by homologous, double antibody radioimmunoassay as proposed by Midgley [8] and Reuter et al. [15]. The FSH

antiserum was a Calbiochem preparation. 125 I-labelled FSH (CIS FSH-125-B) was purified before use as described for LH. The reference FSH standard, the buffer solution and other details of the method were similar to those indicated for LH except that delayed addition of tracer 24 h after an incubation of samples with the first antibody for 24 h at room temperature was followed by a second incubation under similar conditions prior to addition of precipitating antibody, resulted in enhancement of attainable sensitivity [17].

*Prolactin radioimmunoassay*

A homologous, double antibody radioimmunoassay was performed according to Guyda et al. [6] and Aubert [2]. The antibody used was a specific anti-human rabbit serum (Calbiochem). The prolactin isotope preparation of CIS (PROL-125-B) with a 125 I-label was purified before use as described for LH. The prolactin standard was a "MRC Research Standard A for Prolactin Human 71/222" with a biological activity of 1.0 mIU = 100 ng, kindly provided by WHO authorities. The radioassay was performed as described for LH and by a delayed addition of tracer to increase sensitivity as indicated for FSH. Fifty $\mu$l aliquots of unknown serum samples were diluted to 200 $\mu$l with buffer and processed.

## Results

Results of control experiments for reliability criteria of methods are summarized in Table I. The intra- and interassay coefficients of variation were calculated from 20 parallel estimates. Accuracy of methods was controlled by external standards kindly provided by WHO. The results appeared to be comparable to those reported by others.

The fluctuation of serum gonadotropin concentration due to their episodic secretion was studied in 8 healthy women on the 2, 2, 6, 10, 18, 20, 20 and 21

**Table I**

*Reliability criteria of methods*

| | LH | FSH | Prolactin |
|---|---|---|---|
| Sensitivity, mIU/ml | 0.5 | 0.5 | 1.0* |
| Intraassay CV, per cent | 5.5 | 4.6 | 4.7 |
| Interassay CV, per cent | 13.2 | 11.4 | 11.8 |
| Accuracy, per cent | 14.5 | 12.5 | 15.4 |

* ng/ml
CV coefficient of variation

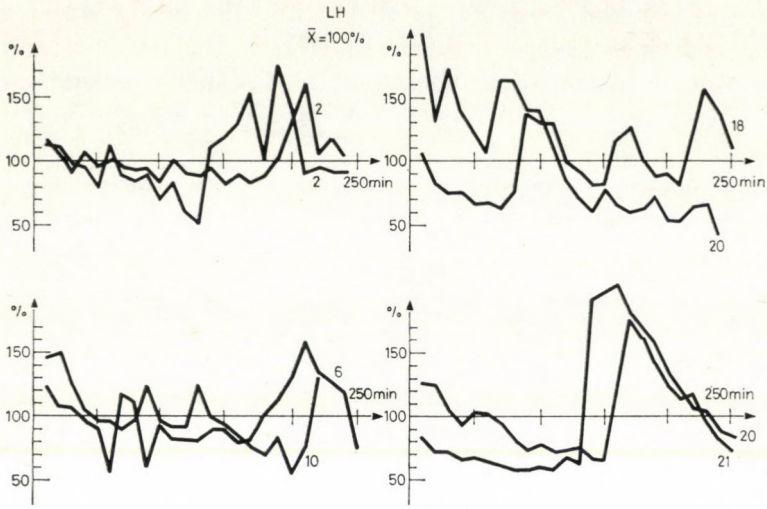



*Fig. 1.* Episodic fluctuation of serum LH level in 8 healthy women. The values are expressed as per cent deviation from the mean of 25 measurements in 10 min intervals during a 4 h period between 8 and 12 h a.m. Horizontal lines represent the mean taken as 100%. The numbers at the end of diagrams indicate the days of menstrual cycle examined

days respectively of their menstrual cycle, in order to investigate differences which may be characteristic of the different phases of the cycle. The absolute values measured reflected a physiological range during the menstrual cycle. To make comparable the great inter-individual differences, the single estimates were expressed as a percent deviation from the mean intra-individual value. The relative fluctuations of individual estimates are shown in Figs 1, 2, and 3.

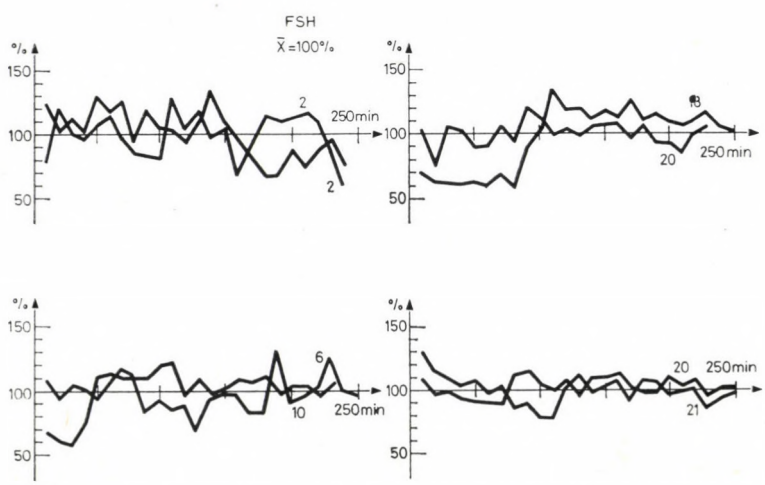



*Fig. 2.* Episodic fluctuation of serum FSH level in 8 healthy women. For details, see Fig. 1

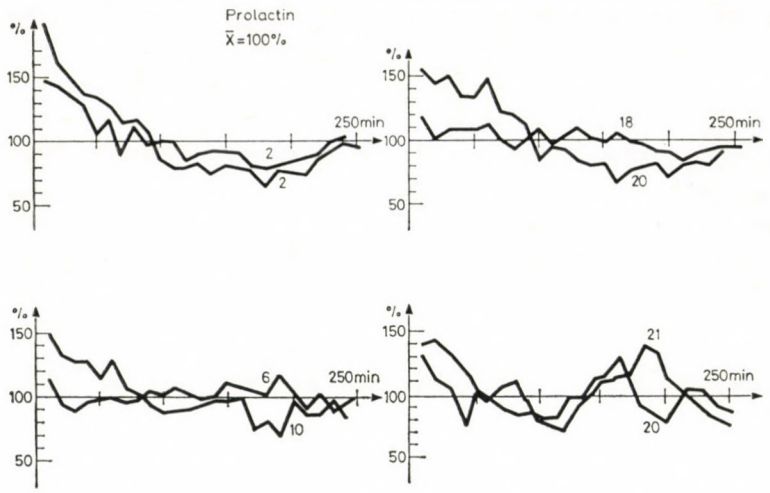



*Fig. 3.* Episodic fluctuation of serum prolactin level in 8 healthy women. For details, see Fig. 1.

The horizontal lines in the Figures represent the mean of 25 estimates which was considered 100%. Oscillation of individual values showed one to 5 secretory surges in the studied subjects. No differences were found in fluctuation of the hormones studied, characteristic of phases of the menstrual cycle. The LH secretory surges seemed however, to be higher and to appear less frequently in the second phase in comparison to the first phase of the menstrual cycle.

Results of statistical evaluation of within-person individual estimates are summarized in Table II. Although the range and the mean of the coefficients of variation of within-person estimates, and the maximum deviation from the mean hormone level, for LH exceeded those calculated for FSH and prolactin. The oscillation pattern of all gonadotropins was found considerable.

**Table II**

*Calculated parameters of fluctuation of gonadotropin levels in serum of 8 menstruating women during a 4 h experimental period (25 estimates)*

| | LH | FSH | Prolactin |
|---|---|---|---|
| Within person | 16.1–48.2* | 7.1–26.3 | 7.4–34.5 |
| CV, per cent | 30.3** | 15.1 | 19.2 |
| Maximum deviation from the mean at 95 per cent CL, per cent | 60.6 | 30.2 | 38.4 |

* range
** arithmetical mean
CV, coefficient of variation; CL, confidential limit

*Significance of methodological error in analysis of episodic fluctuations*

The findings raise the possibility of methodological errors to interfere with the blood levels of hormones. In calculated values for the episodic fluctuation of hormone levels the methodological error is determined by the magnitude of the intraassay coefficient of variation, since determination of gonadotropins was carried out within a single assay. The interrelation between two independent factors may be assessed by the Gauss equation: $S^2 = S_1^2 + S_2^2$, the square resultant standard deviation being equal to the sum of square standard deviation of individual factors.

Taking LH values into consideration, if the observed fluctuation of within-person blood estimates was 30.3%, and the intraassay coefficient of variation for LH radioimmunoassay 5.5%, then the actual (corrected) fluctuation of the hormone level will be 29.8%, which is only 0.5% less than that obtained without correction. According to these calculations, the methodological error in the evaluation of episodic fluctuations can be neglected.

## Discussion

Episodic secretion of LH and FSH was first reported by Yen and Tsai [20], Naukin and Troen [13] and Midgley and Jaffe [10]. In these investigations a more pronounced fluctuation of the LH blood level than that of FSH was observed. This may be explained by a difference in their biological half-life (60 min versus 6 h in healthy women). These differences may also explain the asynchrony of episodic surges of the two gonadotropins. Taking into consideration these estimations, Yen et al. [21] postulated that an intermittent signal from a central neuronal mechanism triggers a periodic discharge of pituitary gonadotropins, which is responsible for their episodic secretion. The frequency and magnitude, however, seem to be influenced by other peripheral hormones.

The aim of the present investigations was to examine the serum LH and FSH oscillations in menstruating women and to consider quantitatively the validity in clinical practice of a single serum hormone estimate. Our observations compare favourably with those of Santen and Bardin [18] and Goldzieher et al. [5]. They found the coefficients of variation of single estimates between 21 and 51%. The significant oscillation, particularly of LH, suggests a need of simultaneous measurements of these hormones. In the evaluation of values, however, the cyclic change of serum gonadotropins, that they exhibit no diurnal variation, and that the episodic secretory surges occur in one to four hour intervals, must also be taken into consideration [9, 21].

Our clinical experience for instance in the differential diagnostics of cases with amenorrhoea, proved a 60% reduction of error in hormone determinations by calculating the mean value of measurements on 3 subsequent days.

A fluctuation of the prolactin concentration in serum was first demonstrated by Sassin et al. [19] and Parker et al. [14]. According to the present investigations, a considerable fluctuation of the blood prolactin concentration was also observed. The initially high values observed may be explained by the stress of examination [1]. The maximum increase in the prolactin level does not occur earlier than 15 to 30 min following a stress, therefore a single blood prolactin determination may yield less misleading information than that for LH or FSH [12]. In our patients it might arise before the 4 h examination when they have already learned the details of the study. In view of the considerable oscillation of prolactin levels, several blood samplings seem better to approach the true mean hormone concentration. In timing blood sampling, the occurrence of secretory surges between 01 and 04 h as well as the circadian variation with a maximum at daybreak [14, 4] must be considered. Thus blood sampling for prolactin assay should be done one to two hours after awakening and on several subsequent days. According to our experience, the mean value obtained at this time on three consecutive days reduced the error with about 60%.

## Acknowledgements

This work was supported in part by the Small Supplies Program of the WHO Special Program of Research, Development and Research Training in Human Reproduction. The authors are indebted to the National Institute of Biological Standards and Control, London, for providing the First International Preparation of Human Pituitary Gonadotropins FSH and LH 69/104 and the MRC Research Standard A for Prolactin Human 71/222. The expert technical assistance of Mrs M. Merényi, Mrs E. Kovács, Mrs M. Tóth and A. Keresztesi is gratefully acknowledged.

## REFERENCES

1. Adler, R. A., Noel, G. L., Wartofsky, L., Frantz, A. G.: Failure of oral water loading and intravenous hypotonic saline to depress plasma prolactin in man. J. Clin. Endocrinol. Metab. **41,** 383–389 (1975)
2. Aubert, M. L.: Radioimmunoassay for human prolactin. In: Abraham G. E. (ed.): Handbook of Radioimmunoassay. Marcel Dekker, Inc. New York 1977, pp. 179–208.
3. Baugham, D. R., Berryman, I., Burger, H., Cotex, M., Furnival B. E., Hunter, W. M., Midgley, A. R., Mussett, M. V., Reichert, L. E., Rosenberg, E., Ryan, R. J., Wide, L.: An international collaborative study of 69/104, a Reference Preparation of Human Pituitary FSH and LH. J. Clin. Endocrinol. Metab. **36,** 647–660 (1973)
4. Djursing, H., Hagen, C., Moller, J., Christiansen, C.: Short- and long-term fluctuations in plasma prolactin concentration in normal subjects. Acta Endocrinol (Copenh.) **97,** 1–6 (1981)
5. Goldzieher, J. W., Dozier, T. S., Smith, K. D., Steinberger, E.: Improving the diagnostic reliability of rapidly fluctuating plasma hormone levels by optimized multiple-sampling techniques. J. Clin. Endocrinol. Metab. **43,** 824–830 (1976)
6. Guyda, H. J., Hwang, P., Friesen, H.: Immunologic evidence for monkey and human prolactin. J. Clin. Endocrinol. Metab. **32,** 120–123 (1971)

7. Midgley, A. R. Jr.: Radioimmunoassay: a method for human chorionic gonadotropin and human luteinizing hormone. Endocrinology **79,** 10–18 (1966)
8. Midgley, A. R. Jr.: Radioimmunoassay for human folliclestimulating hormone. J. Clin. Endocrinol. Metab. **27,** 295–299 (1967)
9. Midgley, A. R. Jr., Jaffe, R. B.: Human luteinizing hormone in serum during the menstrual cycle: determination by radioimmunoassay. J. Clin. Endocrinol. Metab. **26,** 1375–1381 (1966)
10. Midgley, A. R. Jr., Jaffe, R. B.: Regulation of human gonadotropins: X. Episodic fluctuation of LH during the menstrual cycle. J. Clin. Endocrinol. Metab. **33,** 962–969 (1971)
11. Moudgal, N. R., Madhwa Raj: Pituitary gonadotropins. In: Jaffe, B. M. and Behrman, H. R. (eds): Methods of Hormone Radioimmunoassay. Academic Press, New York 1974, pp. 57–85.
12. Moult, P. J. A., Dacie, J., Rees, L., Besser, G. M.: Prolactin pulsatility in patients with gonadal dysfunction. Clin. Endocrinol. (Oxf.) **14,** 387–394 (1981)
13. Naukin, H. R., Troen, P.: Repetitive luteinizing hormone elevations in serum of normal men. J. Clin. Endocrinol. Metab. **33,** 558–560 (1971)
14. Parker, D. C., Rossman, L. G., Vanderlan, E. F.: Sleep-related, nyctohemeral and briefly episodic variation in human plasma prolactin concentrations. J. Clin. Endocrinol. Metab. **36,** 1119–1124 (1973)
15. Reuter, A. M., Ketelslegers, J. M., Hendricks, J. C., Franchimont, P.: Radioimmunoassay of protein hormones: principles and methodology. Hormone Res. **9,** 404–421 (1978)
16. Rodbard, D., Lewald, J. E.: Computer analysis of radioligand assay and radioimmunoassay date. In: Diczfalusy, E. (ed.): Steroid Assay by Protein Binding. Karolinska Symposia on Methods in Reproductive Endocrinology. Karolinska Institutet, Stockholm 1970, pp. 79–103.
17. Rodbard, D., Ruder, H. J., Vaitukaitis, J., Jacobs, H. S.: Mathematical analysis of kinetics of radioligand assays: improved sensitivity obtained by delayed addition of labelled ligand. J. Clin. Endocrinol. Metab. **33,** 343–355 (1971)
18. Santen, R. J., Bardin, C. W.: Episodic luteinizing hormone secretion in man. J. Clin. Invest. **52,** 2617–2628 (1973)
19. Sassin, J. F., Frantz, A. G., Weitzman, E. D., Kapen, S.: Human prolactin: 24-hour pattern with increased release during sleep. Science **177,** 1205–1207 (1972)
20. Yen, S. S. C., Tsai, C. C.: The biphasic pattern in the feedback action of ethinyl estradiol on the release of pituitary FSH and LH. J. Clin. Endocrinol. Metab. **33,** 882–887 (1971)
21. Yen, S. S. C., Vandenberg, G., Tsai, C. C., Parker, D. C.: Ultradian fluctuations of gonadotropins. In: Ferin, M., Halberg, F., Richart, R. M., Vande Wiele, R. L. (eds): Biorhythms and Human Reproduction. John Wiley and Sons, New York 1974, pp. 203–218.

*Acta Medica Hungarica, 41 (4), pp. 203–211 (1984)*

# EPISODIC SECRETION OF HORMONES AND THE DIAGNOSTIC VALUE OF SINGLE BLOOD ESTIMATES II. PROGESTERONE, OESTRADIOL AND OESTRONE*

G. Siklósi, P. Siklós, A. Hintalan, F. Olajos, Z. Marcsek

SECOND DEPARTMENT OF OBSTETRICS AND GYNAECOLOGY, SEMMELWEIS UNIVERSITY MEDICAL SCHOOL, BUDAPEST, HUNGARY

(Received June 9, 1983)

The episodic fluctuation of serum progesterone, oestradiol and oestrone levels was studied. Progesterone was determined in the luteal phase, oestradiol and oestrone concentrations were measured in the proliferative and luteal phases in 8 subjects in 10 min intervals between 08 and 12 h a.m. Reliability criteria proved the radioimmunological methods used to be comparable to those employed routinely. The within-person fluctuation of hormone levels characterized by the coefficient of variation of single estimates averaged 20.16, 23.0 and 11.6% for progesterone, oestradiol and oestrone, respectively. The considerable fluctuation of hormone concentrations suggest the importance of hormone measurements to control luteal function and folliculogenesis during the menstrual cycle.

**Keywords:** Secretion of hormones, progesterone, oestradiol, oestrone.

## Introduction

In the last decade, radioimmunological determination of hormones has been used extensively in clinical diagnostics as well as in research work. It is generally accepted that the circulating progesterone level is characteristic of luteal function [33], that of oestradiol of folliculogenesis [18] and the oestrone level of oestrogen availability in the postmenopausal state and under various experimental conditions [27]. In view of the episodic secretion of these hormones it is a recurrent question in clinical practice how far a single estimate of the serum level reflects the daily mean hormone concentration in blood.

The aim of the present study was to approach quantitatively the rate of fluctuation of the blood progesterone, oestradiol and oestrone concentrations and to determine the representative errors caused by single estimates.

Send offprint requests to G. Siklósi, H-1082 Budapest, Üllői út 78/a, Hungary

* Presented in part at the 9th Congress of the Hungarian Society of Endocrinology and Metabolism, Szeged, Hungary, 1979.

## Patients and methods

Eight apparently healthy women aged 23 to 35 years volunteered for the study. Their weight was ideal with a deviation of at most ±10%. They had regular menstrual cycles of 26 to 30 days. Onset of ovulation and physiological corpus luteum function were controlled by measurement of basal body temperature and serial determination of the serum progesterone level. The episodic fluctuation of blood hormone concentration was studied 90 min. or more after awakening. Blood samples were withdrawn from the cubital vein by an indwelling cannula in 10 min intervals between 08 and 12 h a.m. The 25 blood samples were immediately centrifuged and stored at −20 °C until processing. To exclude interassay methodological errors, all samples of a single series were worked up simultaneously. The mean value of the 25 within-person estimates represented the average blood hormone level. The increment of single estimates from the mean was determined by the coefficient of variation of 25 measurements, and the maximum deviation of individual values from the mean characterized at 95% confidential limits (2 S.D.). To allow comparison of inter-person estimates, the values were expressed in percentage of the intra-person average value.

## Hormone determinations

*Progesterone radioimmunoassay*

Progesterone in serum was determined by no-chromatography charcoal adsorption technique according to Abraham et al. [1, 2, 3, 6], with modifications [8, 9, 19, 30, 36].

The antibody used was raised against progesterone-11α-succinyl-BSA in rabbit (Steranti, England, Code P 001), with a minimum cross reaction with interfering steroids (5α-pregnanediol 2.5%, other disturbing C-21 deoxypregnanes < 1%), allowing an application without chromatography of serum progesterone [8, 19]. 1.2,6,7-$^3$H-progesterone (Amersham, specific activity 3.55 TBq/mmol) was purified every 6 months by means of celite chromatography (Celite 535, Light, England), as described previously [4.28]. Reference progesterone (Sigma Chemicals) was dissolved in ethanol and diluted as indicated below. All dilutions were made by phosphate buffer (0.1 mol, pH 7.0, containing 0.9% NaCl; 0.1% Na azide; and 0.1% gelatin the charcoal suspension contained 0.625 g/dl Norit A [Serva, Heidelberg] and 0.0625 g/dl Dextran T 30 [Pharmacia, Sweden]). Peroxide-free ether was used for extraction. The scintillation fluid for measurement was a solution of 5.0 g PPO and 0.5 g POPOP in 1000 ml toluene and 200 ml dioxan. Radioactivity was measured in a Packard Tri-Carb Model 3380 liquid scintillation spectrometer with an efficiency of 55%. In 10 × 100 mm glass tubes 20.0 μl serum was diluted to 200.0 μl with buffer, in duplicate, and it was extracted with 2.0 ml ether by vortexing.

The sample was frozen at −20 °C for two hours, decanted and the organic phase evaporated. The residue was dissolved in 500 μl buffer incubated at 60 °C for 30 min and vortexed for 30 sec. The efficiency of recovery was checked by intermittent application of tracer, and the results (91.0±3.5%) showed that internal standardization was unnecessary [8]. The mean value of random recovery experiments was applied to correct the methodological loss. Known progesterone samples contained in triplicate 80.0, 160.0, 320.0, 640.0 and 1280.0 pmol authentic standard in 500 μl buffer.

To unknown serum samples and to standards 10 000 cpm tracer in 100 μl buffer and, after vortexing, 100 μl antiserum ($B_0$–N/T 45–55%) were added and incubated at room temperature for 30 min, then at 4 °C for 16 h. For separation of free and bound fraction, 200 μl charcoal suspension was added at 0 °C, vortexed, incubated at 0 °C for 20 min, and centrifuged (4000 rpm) at zero to 4 °C. The supernatant was decanted into a vial, vortexed for 10 sec and the radioactivity was measured. Results were calculated by logit-log transformation in a programmed Hewlett–Packard 67 calculator as proposed by Rodbard et al. [31].

Control experiments revealed the method's sensitivity to be 32.0 pmol/tube (1.6 nmol/l serum). The intra- and interassay coefficients of variation in 20 measurements were 6.7 and 12.9%, respectively. Accuracy of the method by using an external standard supplied by the World Health Organization was found to be 14.0%. The reliability criteria of the method were checked as recommended in the literature [5, 10, 21].

*Oestradiol radioimmunoassay*

Radioimmunological determination of oestradiol was performed by the no-chromatography charcoal separation technique as originally described by Edquist and Johansson [15] and Emment et al. [16] with minor modifications [5, 11, 17, 23, 24, 29, 37].

The antiserum was anti-17$\beta$-oestradiol-6-carboxymethyloxime-BSA serum raised in rabbit (Steranti, England, Code E 002) with a cross-reaction of disturbing steroids <1% [37]. 2,4,6,7-$^3$H-oestradiol (Amersham, specific activity 3.7 TBq/mmol) was purified every 6 months [4, 28]. Authentic oestradiol (Sigma Chemicals) was dissolved in ethanol and further dilutions were made with the buffer solution described above.

For radioassay of unknown sera 200.0 $\mu$l aliquots in duplicate and for the known samples 23.0, 46.0, 92.5, 185.0, 370.0, 740.0 and 1480.0 pmol oestradiol in buffer were used. The method was similar to that described for progesterone with the exception that to increase the method's sensitivity addition of the isotope was delayed [32]. After incubation with the antibody at room temperature for 30 min 10 000 cpm tracer was added at 0 °C followed by another incubation at 4 °C for 16–24 h.

According to control experiments the method's sensitivity was 10 pmol/tube or 50 pmol/l serum. The intraassay coefficient of variation in 20 samples was 6.2% and the interassay coefficient of variation in 10 subsequent assays, 12.4%. An accuracy of 11.7% using the WHO external standard was calculated.

*Oestrone radioimmunoassay*

No-chromatography charcoal technique was employed [12, 16, 29].

A highly specific anti-oestrone-6-carboxymethyloxime-BSA rabbit serum (Steranti, Code E 001) was used. Cross-reaction with whatever oestrogen or disturbing steroid in serum was <1%, as indicated by the maker. 2,4,6,7-$^3$H-oestrone (Amersham, specific activity 3.4 TBq/mmol) was purified by celite chromatography 6-monthly [28]. Authentic oestrone was purchased from Sigma Chemicals.

The radioimmunoassay was performed as described for progesterone. To increase the sensitivity addition of tracer was delayed as indicated for oestradiol [32].

Quality control of assay revealed the method's sensitivity to be 10 pmol/tube or 50 pmol/l serum, an intraassay coefficient of variation (20 samples) of 6.3%, and an interassay coefficient of variation (11 series) of 13.1%.

## Results

### *Fluctuation of serum progesterone concentration*

The episodic fluctuation of progesterone concentration in serum was studied in 8 healthy women at 16, 18, 19, 19, 20, 20, 21, and 26 days, respectively, of their menstrual cycle. Every series of measurements lasted 4 h with samplings every 10 min. The mean values of the 8 series varied between 20.7 and 44.0 nmol/l, all in the physiological range.

To allow an inter-person comparison, the single estimates were expressed as percentage increment of the intra-person mean value, considering the latter to be 100%. Figure 1 shows the rate of fluctuation of the serum progesterone concentration of 8 apparently healthy women. Horizontal lines represent within-person mean values. The within-person coefficients of variation varied from 16.2 to 26.4%, representing a considerable deviation of single estimates from the mean value in a single series. The maximum increment averaged

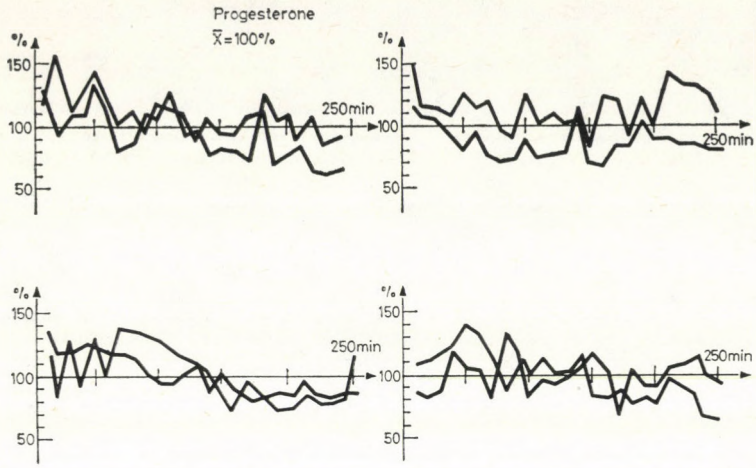



*Fig. 1.* Episodic fluctuation of serum progesterone level in 8 apparently healthy women. The values are expressed as percentage increment of the mean estimate of 25 measurements done at 10 min intervals during a 4 h follow-up study between 08 and 12 h a.m. Horizontal line represents the mean estimate taken as 100%

40.3% at 95% confidential limits (2 SD). Peak values of within-person measurements revealed 3 to 5 secretory surges, their onset was irregular and their intensity inconsistent.

*Fluctuation of serum oestradiol concentration*

The serum oestradiol level was determined in 8 healthy women at 2,2,6,10,18,20,20, and 21 days, respectively, of their menstrual cycle. The mean of within-person estimates varied between 26.4 and 937.6 pmol/l, corresponding to the physiological values. Figure 2 shows the fluctuation of oestradiol estimates in the dimension indicated for progesterone. The coefficients of variation of within-person estimates were between 8.5 and 30.2%, with an average of 23.0% in the 8 series. Average maximum oscillation of hormone concentration in serum at 95% confidential limits was 46.0%. On the basis of peak concentrations, during the 4 h interval 3 to 5 secretory episodes could be identified (Fig. 2). The onset of surges was irregular and their intensity inconsistent.

*Fluctuation of serum oestrone concentration*

Serum oestrone concentration was studied in 8 healthy women at 2,2,6, 10,18,20,20 and 21 days, respectively, of their menstrual cycle. The hormone levels varied in the physiological range exhibiting mean values between 26.4

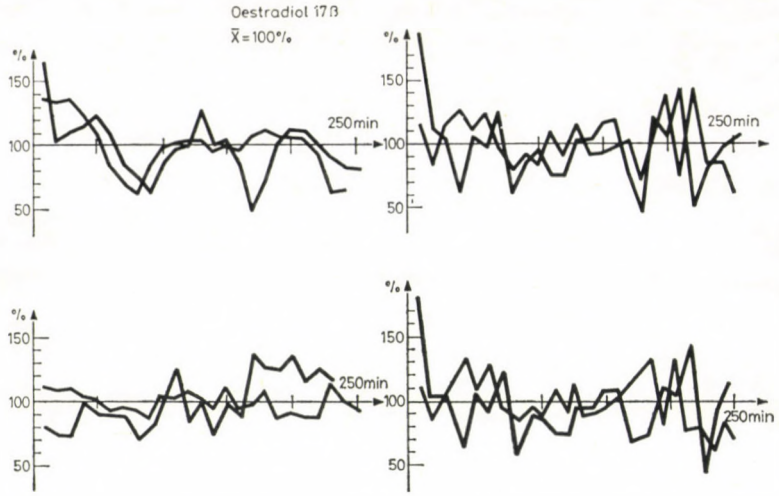



*Fig. 2.* Episodic fluctuation of serum oestradiol level in 8 apparently healthy women. For details, see Fig. 1

**and 937.6 pmol/l (Fig. 3). The within-person coefficients of variation were between 8.0 and 17.8%, with a mean of 11.6% in the 8 series. Maximum increments of individual estimates at 95% limits of confidence averaged 23.2%. Three to 5 episodes of secretion were identified during the experimental period, with inconsistent fluctuations.**

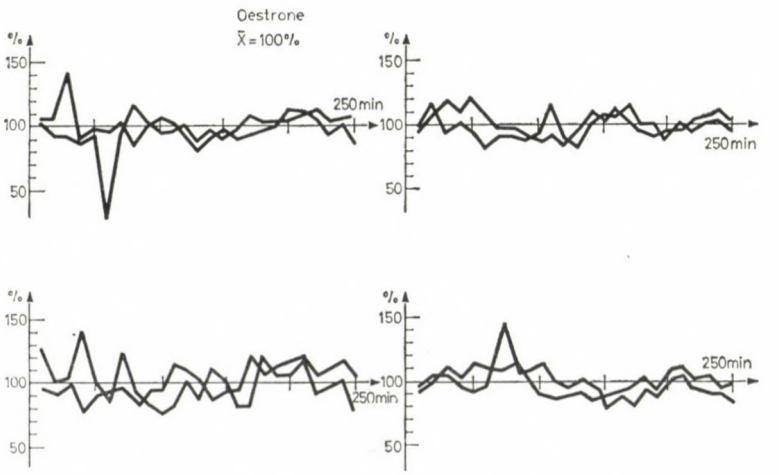



*Fig. 3.* Episodic fluactuation of serum oestrone level in 8 apparently healthy women. For details, see Fig. 1

## Discussion

### *Fluctuation of serum progesterone level*

Oscillation of progesterone concentration in peripheral blood of female subjects was first described by West et al. [35]. To our knowledge, there is no indication in the literature of fluctuation of the daily blood concentration of progesterone. Previous studies showed no diurnal variation in the serum progesterone level similar to that of LH and FSH (25, 26]. Changes of the progesterone level during the menstrual cycle are well documented [20, 22]. These studies carried out on large populations showed progesterone levels below 3 nmol/l (1 ng/ml) in the proliferative phase, a gradual increase after ovulation reaching about 32 nmol/l (10 ng/ml) plateau concentration in the luteal phase, and a subsequent decrease to below 3 nmol/l until menstruation.

Since progesterone determinations are recommended to be performed during the luteal phase the episodic fluctuations were investigated only in this phase in the present study. The aim of serial determinations was to study to what extent an individual progesterone estimate can characterize the mean daily hormone level and how the fluctuation interferes with the assessment of luteal function. According to the present investigation, a single progesterone assay with a mean coefficient of variation of 20.16% and a 40.32% maximum percentage increment would represent the average serum concentration. This indicates that several determinations must be done to control luteal function. Our clinical experience suggested that 60% or higher reduction of errors caused by the oscillation would occur if determinations were performed on 3 different days during the luteal phase, between the 19th and 24th days of the cycle or 4 to 10 days before menstruation.

### *Fluctuation of serum oestradiol level*

In spite of reports on episodic fluctuations of the oestradiol level [7], there is no indication in the literature on the degree of these fluctuations. A diurnal rhythm of serum oestradiol concentration appears to be absent [25]. Changes in the secretory rate during the menstrual cycle have been established [13, 20] and there is a close correlation between serum oestradiol concentration and folliculogenesis [18, 34]. Since determination of the hormone is extensively used to control folliculogenesis and to follow ovarian function after the induction of ovulation, it seems important to know to what extent the daily oscillations lower the value of information supplied by a single hormone measurement. The present findings showed the single oestradiol values to oscillate with a mean coefficient of variation of 23.0%, and the average maximum deviation was 46.0% at 95% limits confidence. This rate of fluctuation indicates that

several hormone measurements are only informative. In cases of amenorrhoea, determination of oestradiol in a pool of sera obtained on several days, while in patients with induction of ovulation, separate determinations are recommended.

*Fluctuation of serum oestrone level*

In the literature no report has been found on pulsatile secretion and episodic fluctuations of the blood oestrone level, nor has a diurnal rhythm been shown [25, 26]. The characteristic change of the serum oestrone level during the menstrual cycle is well-known [20]. Our results showed a fluctuation of oestrone half that observed for oestradiol. The mean coefficient of variation was 11.6% and the maximum increment averaged 23.2% at 95% limits of confidence. Since considerable extraglandular conversion of oestradiol to oestrone occurs [14, 27], the source of variations of the blood oestrone level may be equally glandular and extraglandular.

## Acknowledgements

The work was supported in part by the Small Supplies Program of the WHO Specia Program of Research, Development and Research Training in Human Reproduction. The expert technical assistance of Mrs M. Merényi, Mrs E. Kovács, Mrs M. Tóth and Mrs A. Keresztesi is gratefully acknowledged.

## REFERENCES

1. Abraham, G. E., Swerdloff, R. S., Tulchinsky, D., Odell, W. D.: Radioimmunoassay of plasma progesterone. J. Clin. Endocrinol. Metab. **32,** 619–624 (1971)
2. Abraham, G. E., Hopper, K., Tulchinsky, D., Swerdloff, R. S., Odell, W. D.: Simultaneous measurement of plasma progesterone, 17-hydroxyprogesterone and estradiol-17-beta by radioimmunoassay. Anal. Letter **4,** 325–335 (1971)
3. Abraham, G. E., Odell, W. D., Swerdloff, R. S., Hopper, K.: Simultaneous radioimmunoassay of plasma FSH, LH, progesterone, 17-hydroxyprogesterone and estradiol-17-beta during the menstrual cycle. J. Clin. Endocrinol. Metab. **34,** 312–318 (1972)
4. Abraham, G. E., Buster, J. E., Lucas, L. A., Corrales, P. C., Teller, R.: Chromatographic separation of steroid hormones for use in radioimmunoassay. Anal. Letter. **5,** 509–517 (1972)
5. Abraham, G. E.: Radioimmunoassay of steroids in biological materials. Acta Endocrinol. (Copenh.) Suppl. **183,** 1–42 (1974)
6. Abraham, G. E., Manlimos, F. S., Garza, R.: Radioimmunoassay of steroids. In: Abraham, G. E. (ed.): Handbook of Radioimmunoassay. Marcel Dekker Inc., New York 1977, pp. 591–656.
7. Alford, F. P., Baker, H. W. G., Burger, H. G., de Kretser, D. M., Hundson, E., Johns, M. W., Masterton, J. P., Patel, Y. C., Rennie, G. G.: Temporal patterns of integrated plasma hormone levels during sleep and wakefulness. II. FSH, LH, testosterone and estradiol. J. Clin. Endocrinol. Metab. **37,** 848–854 (1973)
8. Aso, T., Guerro, R., Cekan, Z., Diczfalusy, E.: A rapid 5 hour radioimmunoassay of progesterone and oestradiol in human plasma. Clin. Endocrinol. **4,** 173–182 (1975)
9. Brenner, P. F., Guerro, R., Cekan, Z., Diczfalusy, E.: Radioimmunoassay method for six steroids in human plasma. Steroids **22,** 775–794 (1973)
10. Cekan, Z.: Assessment of reliability of steroid radioimmunoassay. J. Steroid. Biochem. **6,** 271–175 (1975)

11. Doerr, P.: Hapten-radioimmunoassay of plasma oestradiol. Acta Endocrinol (Copenh.) **72,** 330–344 (1973)
12. Doerr, P.: Radioimmunoassay of oestrone in plasma. Acta Endocrinol. (Copenh.) **81,** 655–667 (1976)
13. Dyrenfurth, I., Jewelewicz, R., Warren, M., Ferin, M., Vande Wiele, R. L.: Temporal relationships of hormonal variables in the menstrual cycle. In: Ferin, M., Halberg, F., Richart, R. M., Vande Wiele, R. L. (eds): Biorhythms and Human Reproduction. Wiley and Sons, New York 1974, pp. 171–201.
14. Edman, C. D., MacDonald, D. C.: Effect of obesity on conversion of plasma androstenedione to estrone in ovulatory and anovulatory young women. Am. J. Obstet. Gynecol. **130,** 456–461 (1978)
15. Edquist, L. E., Johansson, E. D. B.: Radioimmunoassay of oestrone and oestradiol in human and bovine plasma. Acta Endocrinol. (Copenh.) **71,** 716–730 (1972)
16. Emment, Y., Collins, W. P., Sommerville, I. F.: Radioimmunoassay of oestrone and oestradiol in human plasma. Acta Endocrinol. (Copenh.) **69,** 567–582 (1972)
17. England, B. G., Niswender, G. D., Midgley, Jr. A. R.: Radioimmunoassay of estradiol-17-beta without chromatography. J. Clin. Endocrinol. Metab. **38,** 42–50 (1974)
18. Freundl, G., Terinde, R., Distler, W., Baumeister, A., Herberger, J. and Kozlowsky, P.: Serum levels of oestradiol-17-beta and luteinizing hormone related to follicular growth as monitored by ultrasound in women with normal menstrual cycles. Acta Endocrinol. (Copenh.) **96,** 370–376 (1981)
19. Garza, R., Burns, B., Manlimos, F., Abraham, G. E.: Radioimmunoassay of plasma progesterone without chromatography. Anal. Letter **8,** 225–232 (1975)
20. Guerro, R., Aso, T., Brenner, P. F., Cekan, Z., Landgren, B. M., Hagenfeldt, K., Diczfalusy, E.: Studies on the pattern of circulating steroids in the normal menstrual cycle. I. Simultaneous assay of progesterone, pregnenolone, dehydroepiandrosterone, testosterone, dihydrotestosterone, androsterone, oestradiol and oestrone. Acta Endocrinol. (Copenh.) **81,** 133–149 (1976)
21. **Hall, P. E.: Round-table discussion on assay design, standardization and within-laboratory quality control. In: Radioimmunoassay and Related Procedures in Medicine 1977. Vol. II. International Atomic Energy Agency, Vienna 1978, pp. 3–79.**
22. **Johansson, D. B., Wide, L., Gemzell, C.: Luteinizing hormone (LH) and progesterone in plasma and LH and oestrogens in urine during** 42 normal menstrual cycles. Acta Endocrinol. (Copenh.) **68,** 502–512 (1971)
23. Jurjens, H., Pratt, J. J., Woldring, M. G.: Radioimmunoassay of plasma estradiol without extraction and chromatography. J. Clin. Endocrinol. Metab. **40,** 19–25 (1975)
24. Korenman, S. G., Stevens, R. H., Carpenter, L. A., Robb, M., Niswender, G. D., Sherman, B. M.: Estradiol radioimmunoassay without chromatography: procedure, validation and normal values. J. Clin. Endocrinol. Metab. **38,** 718–720 (1974)
25. Landgren, B.-M., Aedo, A.-R., Nunez, M., Cekan, Z. C., Diczfalusy, E.: Studies on the pattern of circulating steroids in the normal menstrual cycle. 4. Periovulatory changes in relation to the LH surge. Acta Endocrinol. (Copenh.) **84,** 620–632 (1977)
26. Landgren, B.-M., Campo, S., Cekan, Z. C., Diczfalusy, E.: Studies on the pattern of circulating steroids in the normal menstrual cycle. 5. Changes around the onset of menstruation. Acta Endocrinol. (Copenh.) **86,** 608–620 (1977)
27. MacDonald, P. C., Edman, C. D., Gemzell, D. L., Porter, J. C., Siiteri, P. K.: Effect of obesity on conversion of plasma androstenedione to estrone in postmenopausal women with and without endometrial cancer. Am. J. Obstet. Gynecol **130,** 448–455 (1978)
28. Manlimos, F. S., Abraham, G. E.: Chromatographic purification of steroids prior to use in radioimmunoassay. Anal. Letter **8,** 403–410 (1975)
29. Orczyk, G. P., Caldwell, B. V., Behrman, H. R.: Estrogens: estradiol, estrone and estriol. In: Jaffe, B. M., Behrman, H. R. (eds): Methods of Hormone Radioimmunoassay Academic Press, New York 1974, pp. 333–345.
30. Orczyk, G. P., Hichens, M., Arth, G., Behrman, H. R.: Progesterone. In: Jaffe, B. M., Behrman, H. R. (eds): Methods of hormone radioimmunoassay. Academic Press, New York 1974, pp. 347–358.
31. Rodbard, D., Lewald, J. E.: Computer analysis of radioligand assay and radioimmunoassay date. In: Diczfalusy, E. (ed.): Steroid Assay by Protein Binding. Karolinska Symposia on Methods in Reproductive Endocrinology. Karolinska Institutet, Stockholm 1970, pp. 79–103.
32. Rodbard, D., Ruder, H. J., Vaitukaitis, J., Jacobs, H. S.: Mathematical analysis of kinetics of radioligand assays: improved sensitivity obtained by delayed addition of labeled ligand. J. Clin. Endocrinol. Metab. **33,** 343–355 (1971)

33. Rosenfeld, D. L., Chudow, S., Bronson, R. A.: Diagnosis of luteal phase inadequacy. Obstet. Gynecol. **56,** 193–196 (1980)
34. Smith, D. H., Picker, R. H., Sinosich, M., Saunders, D. M.: Assessment of ovulation by ultrasound and estradiol levels during spontaneous and induced cycles. Fertil. Steril. **33,** 387–390 (1980)
35. West, C. D., Mahajan, D. K., Chavré, V. J., Nabors, C. J., Tyler, F. H.: Simultaneous measurement of multiple plasma steroids by radioimmunoassay demonstrating episodic secretion. J. Clin. Endocrinol. Metab. **36,** 1230–1236 (1973)
36. De Villa, G. O. Jr., Roberts, K., Wiest, W. G., Mikhail, G., Flickinger, G.: A specific radioimmunoassay of plasma progesterone. J. Clin. Endocrinol. Metab. **35,** 458–460 (1972)
37. Wu, C.-H., Lundy, L. E., Lee, S. G. L.: A rapid radioimmunoassay for plasma estrogen. Am. J. Obstet. Gynecol. **114,** 169–180 (1973)

*Acta Medica Hungarica 41, (4), pp. 213–222 (1984)*

# EPISODIC SECRETION OF HORMONES AND THE DIAGNOSTIC VALUE OF SINGLE BLOOD ESTIMATES III. TESTOSTERONE, ANDROSTENEDIONE, DEHYDROEPIANDROSTERONE, DEHYDROEPIANDROSTERONE SULPHATE, CORTISOL*

G. Siklósi, A. Hintalan, S. Csömör jr., L. Bakos, P. Siklós, F. Olajos, Z. Marcsek

SECOND DEPARTMENT OF OBSTETRICS AND GYNAECOLOGY SEMMELWEIS UNIVERSITY MEDICAL SCHOOL, BUDAPEST

(Received June 9, 1983)

The episodic fluctuation of serum testosterone, androstenedione, dehydroepiandrosterone (DHA), dehydroepiandrosterone sulphate (DHAS) and cortisol levels was analysed to determine the reliability of a single blood estimate in characterizing the mean value of a 4 h period. Radioimmunoassay of the steroids was performed in sera of 8 apparently healthy women in various phases of the menstrual cycle in 10 min intervals between 08 and 12 h a.m. Reliability criteria of the methods used were comparable to those in common use. The within-person fluctuation of individual values was determined by the coefficient of variation of single estimates, and the methodological error of the single estimates was characterized with the maximum increment from the mean at 95% confidential limits. A maximum deviation of 30.8, 28.6, 37.0, 25.0 and 61.2%, respectively, found in the above order of steroids, suggests several hormone estimations to be necessary for judging hormone availability in a subject.

**Keywords:** Secretion of hormones, testosterone, androstenedione, dehydroepiandrosterone, dehydroepiandrosterone sulphate, cortisol.

## Introduction

The introduction of sensitive radioimmunoassays of steroid hormones led to a better understanding of hyperandrogenic states of women and disturbances of the reproductive system [27, 33]. Due to a pulsatile release and subsequent oscillation of blood hormone levels may, however result in differences of the single estimates from the mean value characterizing the hormone homeostasis.

The present work was undertaken to determine quantitatively the hitherto scarcely studied oscillation of androgen and preandrogen concentrations and that of cortisol in peripheral blood.

Send offprint requests to G. Siklósi, Üllői út 78/a, H-1082 Budapest, Hungary

* Presented in part at the 9th Congress of the Hungarian Society of Endocrinology and Metabolism, Szeged, Hungary, 1979.

## Patients and methods

Eight apparently healthy women aged 23 to 35 years volunteered in the present study. Their weight was ideal with a deviation of ±10%. They had regular menstrual cycles of 26 to 30 days duration. Onset of ovulation and physiological corpus luteum function were controlled by measurement of basal body temperature and serial determinations of serum progesterone concentration. Blood samples were withdrawn by an indwelling cannula from forearm vein 90 min or more after awakening every 10 min from 08 to 12 h a.m. The 25 samples were immediately centrifuged and stored at −20 °C until processing. To exclude interassay methodological error, samples of a single series were worked up simultaneously. The mean value of the 25 within-person estimates represented the informative blood hormone level. The deviation of single estimates from the mean was determined by the coefficient of variation (standard deviation expressed in percent) of 25 measurements, and the maximum increment within individual values was characterized by the 95% confidential limits (2 S.D.). To allow comparison of interperson estimates, the values were expressed in percentage increments from the intraperson average value.

## Hormone determinations

### *Testosterone radioimmunoassay*

Testosterone in serum was determined by no-chromatography charcoal adsorbtion technique as described by Furuyama et al. [16] with modifications [3, 4, 7, 14, 22, 25, 28].

An antiserum raised against testosterone-3-O-carboxymethyloxime-BSA in sheep code S-1599/3 (kindly provided by Prof. G. E. Abraham, Torrance, California) was used. A cross-reaction of 67% with dihydrotestosterone and one of <1% with whatever steroid were indicated. This allowed a determination of "total" testosterone including some dihydrotestosterone [22, 25, 30]. 1,2,6,7-$^3$H-testosterone purchased from Amersham (specific activity 4.07 TBq/mmol) was purified with celite chromatography every 6 months [1, 26]. Reference testosterone (Sigma Chemicals) was dissolved in ethanol and diluted with buffer. All dilutions were made with phosphate buffer (0.1 mol pH 7.0 containing 0.9% NaCl, 0.1% Na azide and 0.1% gelatin). The charcoal suspension contained 0.625% Norit A (Serva) and 0.0625% dextran T 70 (Pharmacia). Peroxide-free ether was used for extraction. The scintillation fluid for radioactivity measurements was a solution of 5.0 g PPO and 0.5 g POPOP in 1000 ml toluene + 200 ml dioxan. Radioactivity was measured in a Packard Tri-Carb Model 3380 liquid scintillation spectrometer with an efficiency of 55%.

### *Method*

In 10 × 100 mm glass-tubes 100 µl serum was extracted with 2.0 ml ether by vortexing in duplicate. Following evaporation the extract was dissolved in 500 µl buffer. The efficiency of extraction was controlled in each series by using tracer in quadruplicate. Since a recovery of 90.0 ± 4.1% (mean ± S.D.) was calculated, internal standardization was regarded as unnecessary.

To 100 µl unknown serum extract in duplicate and to testosterone standards containing 43.5, 87.0, 175.0, 350.0, and 700.0 pmol of the steroid in triplicate, 10 000 cpm tracer, and after vortexing, 100 µl antiserum ($B_0$–N/T 45 to 55%) were added. The samples were incubated at room temperature for 30 min, then at 4 °C for 16 to 24 h. Free and bound fractions were separated by the addition of 200 µl charcoal suspension at 0 °C, vortexed, incubated at 0 °C for 20 min and centrifuged at 4000 rpm/min at 0 °C. The supernatant containing the bound fraction was decanted into a vial, 10 ml scintillation fluid was added and the radioactivity measured. Results were calculated by logit-log transformation in a programmed Hewlett–Packard 67 calculator as proposed by Rodbard et al. [31].

Control experiments showed the method's sensitivity be 10.5 to 21.0 pmol/tube (105 to 210 pmol/ml serum). The intraasssay coefficient of variation (20 samples) was 5.8% and the interassay coefficient of variation (14 series) was 12.6%. Accuracy of the method was 13.5% with the external standard supplied by WHO. These parameters of reliability were calculated as recommended by Abraham [3], Cekan [13] and Hall [20].

*Androstenedione radioimmunoassay*

In the radioimmunological determination of androstenedione the method of Abraham [3] and Parker et al. [29] was followed.

The antiserum was anti-androstenedione-3-carboxymethyloxime-HSA sheep serum, and the only major cross-reactions with physiological compounds were: etiocholanolone 10% and androsterone 4.6% (Prof. G. E. Abraham, Torrance, California, code S-1600) [2]. 1,2,6,7-$^3$H-androstenedione (Amersham, specific activity 3.18 TBq/mmol) was purified on celite column every 6 months. Authentic androstenedione was purchased from Sigma Chemicals, and the reference standard dilutions contained 43.8, 87.5, 175.0, 350.0, 700.0, 1400.0, and 2800.0 pmol of the steroid. Two 50 $\mu$l aliquots of serum were used for determination of unknown samples. The method was identical to that described for testosterone. The mean value of random recovery experiments was 88.2$\pm$3.3% (mean $\pm$ S.D.). No corrections were made according to methodological loss.

Sensitivity of the method was 17.5 to 28.0 pmol/tube (350 to 560 pmol/ml serum). The intra- and interassay coefficients of variation were 6.6 and 13.6% respectively.

*Dehydroepiandrosterone radioimmunoassay*

The method of Buster and Abraham [10, 11] was followed. The antiserum was an anti-DHA-3-hemisuccinate-HSA sheep serum. A 100% cross-reaction with DHAS, 2.3% with androsterone, and one below 1% with other steroids were determined. 1,2,6,7-$^3$H-dehydroepiandrosterone (Amersham, specific activity 3.63 TBq/mmol), authentic DHA (Sigma Chemicals in a dilution of 87.5, 175.0, 350.0, 700.0, 1400.0, and 2800.0 pmol were used. Two 20 $\mu$l aliquots of the serum were diluted to 100 $\mu$l and processed as indicated for testosterone.

Quality control of assay showed the method's sensitivity to be 17.5 to 28.0 pmol/tube (875 to 1400 pmol/ml serum). For the intra- and interassay coefficients of variation, 7.8 and 14.6% respectively, were calculated.

*Dehydroepiandrosterone sulphate radioimmunoassay*

The non-extraction technique recommended by Buster and Abraham [12] was employed. The antiserum and isotope were identical to those used for the parent unconjugated hormone. A calibration between 87 and 2780 pmol authentic DHAS (Sigma Chemicals), and 0.1 and 0.5 $\mu$l of serum samples in duplicate were processed.

According to control experiments, the sensitivity was 17 to 28 pmol/tube (0.1 to 2.8 $\mu$mol/ml or 0.8 to 1.4 $\mu$mol/ml serum, depending on the volume of serum processed). An intra-assay coefficent of variation of 7.2% and an interassay coefficient of variation of 14.5% were calculated.

*Cortisol radioimmunoassay*

Radioimmunological determination of cortisol was performed by the no-chromatography extraction technique as originally described by Abraham et al. [2], Garza and Abraham [17] and Vecsei [35].

The antiserum was raised against cortisol-21-monosuccinate-HSA in shep (Prof. G. E. Abraham, Torrance, California, code S-6[3]). A cross-reaction of 12% with cortisone and 9% with deoxycortisol validated its use for routine purposes. 1,2,6,7-$^3$H-cortisol (Amersham, specific activity 3.0 TBq/mmol) and authentic cortisol (Sigma Chemicals) in dilutions between 3.5 and 56.0 nmol were employed.

Cortisol from 50 $\mu$l serum samples after dilution to 100 $\mu$l was extracted with dichloromethane and processed as described above for testosterone. Mean random recovery was 87.6$\pm$4.3%. The use of internal standard in each sample was disregarded. If in the samples a low cortisol concentration was expected or found, 100 $\mu$l serum was processed.

Sensitivity of the method was 0.56 nmol/tube (corresponding to 11.2 nmol/ml serum), the intra- and interassay coefficients of variation of 6.8% and 13.6%, respectively, and an accuracy with WHO external standard of 14.2% proved the reliability of the method.

## Results

### *Fluctuation of serum testosterone level*

The episodic fluctuation of testosterone concentration in serum was studied in 8 women on the 2nd, 2nd, 6th, 10th, 18th, 20th, 20th and 21st days, respectively, of the menstrual cycle. The individual hormone levels averaged 1099 to 2392 pmol/l, corresponding to the physiological range of healthy women. Figure 1 shows the within-person fluctuation of 25 single estimates in percentage of the mean value during the 4 h study period. Horizontal lines represent 100%. The coefficients of variation characterizing the rate of within-person fluctuation varied between 10.2 and 23.1% (mean, 15.4%). The maximum increments in the individual series averaged 30.8% at 95% confidential limits (2 S.D.). Peak values of within-person measurements revealed two to 5 secretory surges. Their onset was irregular and their intensity inconsistent.

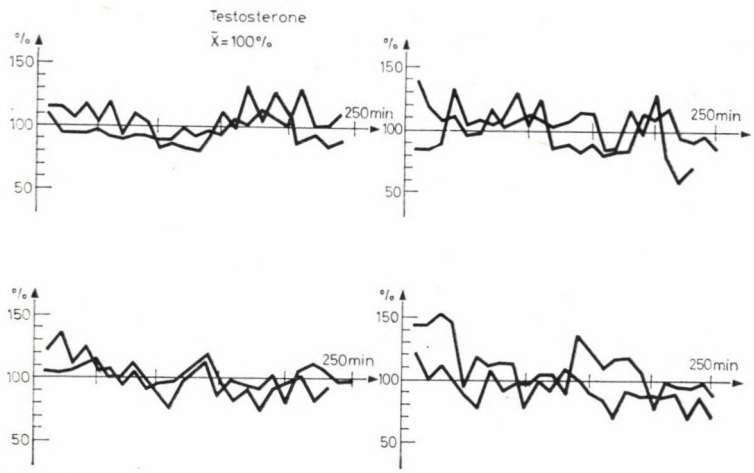



*Fig. 1.* Episodic fluctuation of serum testosterone level in 8 healthy women. The values are expressed as percentage deviation from the mean of 25 measurements at 10 min intervals during a 4 h study period between 08 and 12 h a.m. Horizontal line represents the mean estimate taken as 100%

### *Fluctuation of serum androstenedione level*

The oscillation of androstenedione concentration in serum of 8 women on the 2nd, 2nd, 6th, 10th, 18th, 19th, 20th and 26th day of their menstrual cycle is shown on Fig. 2. The single estimates were evaluated as for testosterone. The individual hormone levels averaged 4.12 to 6.95 nmol/l, all within the normal range. The coefficients of variation representing the rate of within-person

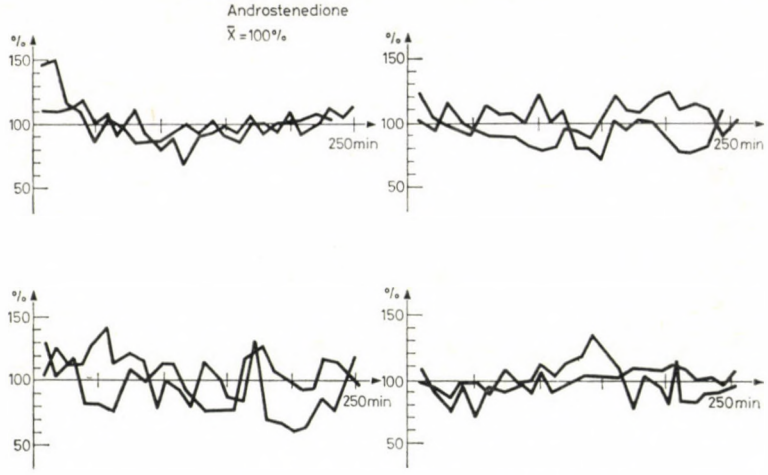



*Fig. 2.* Episodic fluctuation of serum androstenedione level in 8 healthy women. For details, see Fig.1

fluctuation of androstenedione values in 8 subjects were between 8.9 and 20.3% (mean, 14.3%). The individual maximum increments averaged 28.6% at 95% confidential limits. As it can be seen in Fig. 2, 2 to 5 secretory surges could be identified from the blood hormone levels. Their onset and intensity were inconsistent.

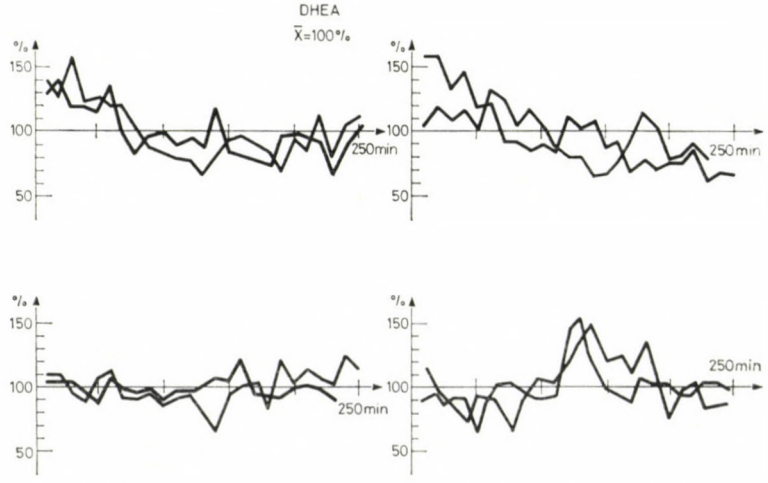



*Fig. 3.* Episodic fluctuation of serum dehydroepiandrosterone level in 8 healthy women. For details, see Fig. 1

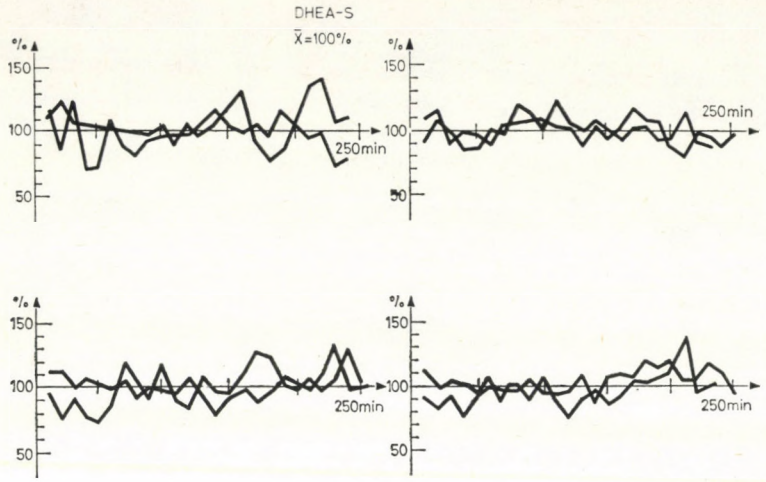



*Fig. 4.* Episodic fluctuation of serum dehydroepiandrosterone sulphate level in 8 healthy women. For details, see Fig. 1

### *Fluctuation of serum dehydroepiandrosterone level*

It was studied in 8 apparently healthy women on the 2nd, 2nd, 6th, 10th, 18th, 20th, 20th and 26th day of their menstrual cycle (Fig. 3). The hormone values for single subjects averaged 11.75 to 24.10 nmol/l, and the variation coefficients of within-person single estimates averaged 7.2 to 25.3% (mean, 18.5%). For maximum increments at 95% confidential limits a mean value of 37.0% was calculated. Again 2 to 5 secretory episodes were observed of inconsistent frequency and intensity.

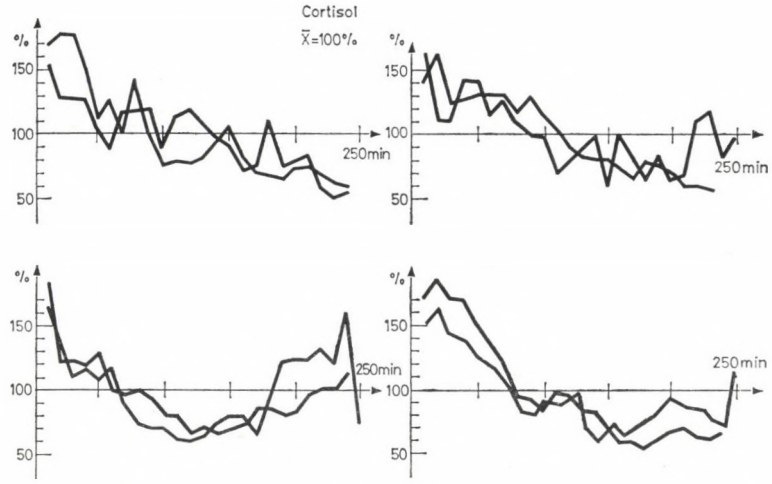



*Fig. 5.* Episodic fluctuation of serum cortisol level in 8 healthy women. For details, see Fig. 1

*Fluctuation of serum dehydroepiandrosterone sulphate level*

The same serum samples as for testosterone were processed for DHAS. The mean hormone concentrations in 8 women were between 3.82 and 7.22 μmol/l. The coefficients of variation reflecting the within-person fluctuation of hormone levels averaged 9.7 to 16.2% (mean, 12.5%). The maximum increment for the 8 subjects was 25.0%. The individual graphs on Figure 4 show 2 to 5 secretory surges, the intensity of which was irregular.

*Fluctuation of serum cortisol level*

Oscillation of blood cortisol concentration during the 4 h period was analysed in the same samples as for testosterone and DHAS. The mean cortisol level for the 8 subjects varied in the physiological range between 184.3 and 417.2 nmol/l. The fluctuation of the 10 min estimates characterized by within-person coefficients of variation exhibited a range between 21.5 and 43.2% (mean, 30.6%). The maximum fluctuation at 95% confidential limits averaged 61.2%. Two to 5 irregular secretory episodes could be identified (Fig. 5). As it can be seen from the graphs, the initial serum cortisol value exceeded at most 40% of the mean intra-person estimates and reached them only after 50 to 110 min.

## Discussion

The first report on discontinous secretion of hormones was published for cortisol by Weitzman et al. [36]. Later their study of hormone secretion was extended to various periods of the day, and they demonstrated that the diurnal change of hormone concentration in peripheral blood was determined by the number and intensity of secretory episodes [37]. The oscillation of DHA level and the synchronism of its secretion with that of cortisol were studied by Rosenfeld et al. [32].

They suggested that the difference in the two hormone levels is a consequence of their different rate of secretion and altered metabolism. The less pronounced fluctuation of DHAS concentration could be explained with its longer half-time [33]. Secretory surges for testosterone were first demonstrated by West et al. [38], it has than been extensively studied [19] and was also observed in hyperandrogenic conditions [23].

Evaluation of hormone estimates in clinical practice has made it necessary to study the fluctuations of serum hormone levels and to determine quantitatively the representative error connected with single estimates. Our measurements during a 4 h period showed the error at 95% limits of confidence to be ±61.2% for cortisol, 37.0% for DHA, 30.8% for testosterone, 28.6% for

androstenedione and 25.0% for DHAS, due to the fluctuation of their blood concentration.

Differences in the rate of fluctuation of serum hormone concentrations appeared to result from differences in the dynamics of their secretion, interconversion, reversible or irreversible metabolism, rate of elimination, etc. [8, 18, 33]. A measure of these metabolic features is the biological half-life, which was about 60 min for cortisol [37], 20 min for DHA [32], 36 min for testosterone [21] and 10 to 20 h for DHAS [33]. The greatest fluctuations of cortisol concentration seem to be, in addition to the metabolic parameters, a consequence of individual stress situations. This was best shown by the initial values of hormone measurements as they exceeded all estimates during the 4 h study period.

The significant amplitudes of fluctuation of the hormone levels observed in the present work suggest the need of simultaneous hormone measurements to approach the actual daily mean blood hormone concentration. Furthermore, to judge the condition of a patient, the diurnal rhythm and the variations of hormone secretion during the menstrual cycle must also be taken into consideration [5, 6, 15, 24, 37].

The present observations suggest that if hormone analysis is performed on 3 subsequent days between 07 and 10 h a.m. and the average value is considered, the methodological error due to fluctuation of the serum hormone level can be reduced by almost 60%. A single estimate of the 3 day serum-pool seems to be equally suitable for routine laboratory diagnostics.

## Acknowledgements

This work was supported in part by the Small Supplies Program of the WHO Special Program of Research, Development and Research Training in Human Reproduction. The authors are indebted to Prof. G. E. Abraham, Harbor General Hospital, Torrance, California for kindly providing the testosterone, androstenedione, DHA and cortisol antisera. The expert technical assistance of Mrs M. Merényi, Mrs E. Kovács, Mrs A. Keresztesi, and Mrs M. Tóth is gratefully acknowledged.

## REFERENCES

1. Abraham, G. E., Buster, J. E., Lucas, L. A., Corrales, P. C., Teller, R. C.: Chromatographic separation of steroid hormones for use in radioimmunoassay. Anal. Letters **5**, 509–517 (1972)
2. Abraham, G. E., Buster, J. E., Teller, R. C.: Radioimmunoassay of plasma cortisol. Anal. Letters **5**, 757–765 (1972)
3. Abraham, G. E.: Radioimmunoassay of steroids in biological materials. Acta Endocrinol. (Copenh.) Suppl. **183**, 1–42 (1974)
4. Abraham, G. E., Manlimos, F. S.: Radioimmunoassay of steroids. In: Abraham, G. E. (ed.): Handbook of Radioimmunoassay. Marcel Dekker, New York 1977, pp. 591–656.
5. Aedo, A.-R., Nunez, M., Landgren, B. M., Cekan, S. Z., Diczfalusy, E.: Studies on the pattern of circulating steroids in the normal menstrual cycle. 3. Circadian variation in the periovulatory period. Acta Endocrinol. (Copenh.) **84**, 320–332 (1977)
6. Aedo, A.-R., Landgren, B.-M., Diczfalusy, E.: Studies on ovarian and adrenal steroids at different phases of the menstrual cycle. II. A comparative assessment of the circadian

variation in steroid and lutropin levels during the follicular, periovulatory and luteal phases. Contraception **23,** 407–424 (1981)
7. Auletta, F. J., Caldwell, B. V., Hamilton, G. L.: Androgens: testosterone and dihydrotestoterone:. In: Jaffe, B. M., Behrman, R. (eds): Methods of Hormone Radioimmunoassay. Academic Press, New York 1974, pp. 359–370.
8. Baird, D. T., Horton, R., Longcope, C., Tait, J. F.: Steroid dynamics under steady-state conditions. Rec. Progr. Horm. Res. **25,** 611–664 (1969)
9. Brenner, P. F., Guerro, R., Cekan, S. Z., Diczfalusy, E.: Radioimmunoassay method for six steroids in human plasma. Steroids **22,** 775–794 (1973)
10. Buster, J. E., Abraham, G. E.: Radioimmunoassay of plasma dehydroepiandrosterone. Anal. Letters **5,** 203–215 (1972)
11. Buster, J. E., Abraham, G. E.: Simultaneous measurement of plasma dehydroepiandrosterone and 16-alfa-dehydroepiandrosterone by radioimmunoassay. Anal. Letters **5,** 597–603 (1972)
12. Buster, J. E., Abraham, G. E.: Radioimmunoassay of plasma dehydroepiandrosterone sulphate. Anal. Letters **5,** 543–551 (1972)
13. Cekan, S. Z.: Assessment of reliability of steroid radioimmunoassays. J. Steroid Biochem. **6,** 271–275 (1975)
14. Coyotupa, J., Parlow, A. F., Abraham, G. E.: Simultaneous radioimmunoassay of plasma testosterone and dihydrotestosterone. Anal. Letters **5,** 329–340 (1972)
15. Frölich, M., Brand, E. C., Van Hall, E. V.: Serum levels of unconjugated aetiocholanalone, androstenedione, testosterone, dehydroepiandrosterone, aldosterone, progesterone and oestrogens during the normal menstrual cycle. Acta Endocrinol. (Copenh.) **81,** 548–562 (1976)
16. Furuyama, S., Mayes, D. M., Nugent, C. A.: A radioimmunoassay for plasma testosterone. Steroids **16,** 415–428 (1970)
17. Garza, R., Abraham, G. E.: Comparison between two assays for plasma cortisol. Anal. Letters **5,** 767–771 (1972)
18. Givens, J. R., Andersen, R. N., Ragland, J. B., Wiser, W. L., Umstot, E. S.: Adrenal function in hirsutism I. Diurnal change and response of plasma androstenedione, testosterone, 17-hydroyprogesterone, cortisol, LH and FSH to dexamethasone and 1/2 unit of ACTH. J. Clin. Endocrinol. Metab. **40,** 988–1000 (1975)
19. Goldzieher, J. W., Dozier, T. S., Smith, K. D., Steinberger, E.: Improving the diagnostic reliability of rapidly fluctuating plasma hormone levels by optimized multiple sampling techniques. J. Clin. Endocrinol. Metab. **43,** 824–830 (1976)
20. Hall, P. E.: Radioimmunoassay and Related Procedures in Medicine 1977. International Atomic Energy Agency, Vienna 1978, pp. 3–79.
21. Horton, R., Shinsko, J., Forham, P. H.: Testosterone production and metabolic clearance rates with volumes of distribution in normal adult men and women. Acta Endocrinol. (Copenh.) **48,** 446–548 (1965)
22. Ismail, A. A., Niswender, G. D., Midgley, A. R. Jr.: Radioimmunoassay of testosterone without chromatography. J. Clin. Endocrinol. Metab. **34,** 177–184 (1972)
23. Ismail, A. A., Davidson, D. W., Souka, A. R., Barnes, E. W., Irvine, W. J., Kilimnik, H. , Vanderbeeken, Y.: The evaluation of the role of androgens in hirsutism and the use of a new anti-androgen "cyproterone acetate" for therapy. J. Clin. Endocrinol. Metab. **39,** 81–95 (1974)
24. Judd, H. L., Yen, S. S. C.: Serum androstenedione and testosterone levels during the menstrual cycle. J. Clin. Endocrinol. Metab. **36,** 475–481 (1973)
25. Lox, C., Christian, C. D., Heine, M. W.: A simple radioimmunoassay for testosterone. Am. J. Obstet. Gynecol. **118,** 114–118 (1974)
26. Manlimos, F. S., Abraham, G. E.: Chromatographic purification of tritiated steroids prior to use in radioimmunoassay. Anal. Letters **8,** 403–410 (1975)
27. Maroulis, G. B., Manlimos, F. S., Abraham, G. E.: Comparison between urinary 17-ketosteroids and serum androgens in hirsute patients. Obstet. Gynecol. **49,** 454–460 (1977)
28. Nieschlag, E.: Radioimmunologische Bestimmung von Testosteron. In: Breuer H., Hamel, D., Krüskemper, H. L. (eds): Methoden der Hormonbestimmung. Thieme Verlag, Stuttgart 1975, pp. 285–292.
29. Parker, L. N., Grover, P. K., Odell, W. D.: Improved radioimmunoassay for 4-androstene-3,17-dione. Steroids **29,** 715–724 (1977)
30. Paulson, J. D., Keller, D. W., Wiest, W. G., Warren, J. C.: Free testosterone concentration in serum: elevation is the hallmark of hirsutism. Am. J. Obstet. Gynecol. **128,** 851–857 (1977)

31. Rodbard, D., Lewald, J. E.: Computer analysis of radioligand assay and radioimmunoassay data. In: Diczfalusy, E. (ed.): Steroid Assay by Protein Binding. Karolinska Symposia on Methods in Reproductive Endocrinology. Karolinska Institutet, Stockholm 1970, pp. 79–103.
32. Rosenfeld, R. S., Hellman, L., Roffwarg, H., Weitzmann, E. D., Fukushima, D. K., Gallagher, T. F.: Dehydroepiandrosterone is secreted episodically and synchronously with cortisol by normal men. J. Clin. Endocrinol. Metab. **33,** 87–92 (1971)
33. Rosenfeld, R. S., Rosenberg, B. J., Fukushima, D. K., Hellmann, L.: 24-hour secretory pattern of dehydroepiandrosterone and dehydroepiandrosterone sulfate. J. Clin. Endocrinol. Metab. **40,** 850–855 (1975)
34. Schulter, D., Burstein, S., Cooke, B. A.: Molecular endocrinology of the steroid hormones. Wiley and Sons, London 1976, pp. 5–55.
35. Vecsei, P.: Glucocorticoids: cortisol, corticosterone, and compound S. In: Jaffe, B. M., Behrman R. (eds): Methods of Hormone Radioimmunoassay. Academic Press, New York 1974, pp. 393–415.
36. Weitzman, E. D., Schaumberg, H., Fishbein, W.: Plasma 17-hydroxycorticoid levels during sleep in man. J. Clin. Endocrinol. Metab. **26,** 121–127 (1966)
37. Weitzman, E. D., Fukushima, D., Nogeire, C., Roffwarg, H., Gallagher, T. F., Hellmann, L.: Twenty-four hour pattern of episodic secretion of cortisol in normal subjects. J. Clin. Endocrinol. Metab. **33,** 14–22 (1971)
38. West, C. D., Mahajan, D. K., Chavré, V. J., Nabors, C. J., Tyler, F. H.: Simultaneous measurement of multiple plasma steroids by radioimmunoassay demonstrating episodic secretion. J. Clin. Endocrinol. Metab. **36,** 1230–1236 (1973)

*Acta Medica Hungarica, 41 (4), pp. 223—227 (1984)*

# A CASE OF 44,X STREAK GONAD SYNDROME COMBINED WITH FAMILIAL BALANCED 13/14 TRANSLOCATION

J. László,[1] P. Bősze,[1] Magdolna Gaál,[2] A. Tóth[1]

[1]DEPARTMENT OF OBSTETRICS AND GYNAECOLOGY, POSTGRADUATE MEDICAL SCHOOL, AND [2]INSTITUTE OF PHYSICAL EDUCATION AND SPORT, BUDAPEST, HUNGARY

(Received April 16, 1983)

A case of 44,X streak gonad syndrome combined with familial balanced 13/14 translocation is described. To our knowledge this is the second report on the combination of X monosomy and autosomal translocation. There was no convincing evidence of an interchromosomal effect in the present case.

**Keywords:** streak gonad syndrome, X monosomy, familial 13/14 balanced translocation

## Introduction

The coexistence of aneuploidy — mostly trisomy — and familial balanced translocation involving unrelated chromosomes has been reported in several cases. Trisomy with unrelated translocation was also found in abortuses [5, 10]. 45,X Turner's syndrome combined with familial balanced 1/2 translocation was described by Kondo et al. [6].

Here we report a case of 44,X streak gonad syndrome [1] and a simultaneous familial balanced 13/14 translocation.

## Report of a case

B. M., aged 23, was first seen at the age of 15 because of short stature and sexual infantilism. She was born at term after an uncomplicated pregnancy and delivery to a 21 year-old healthy mother and 23-year-old healthy father. Birth weight was 2800 g. Her neonatal development was moderately delayed. In her childhood she had suffered from recurrent attacks of otitis and cystopyelitis. She has never had spontaneous menstruation. From the age of 15 she has been on hormonal replacement therapy (oestrogen supplemented with progestins) which resulted in cyclic uterine bleeding, moderate maturation of genitalia and secondary sexual characteristics, and an 8 cm gain in length.

The family history was unremarkable. There is no apparent consanguinity. The proband has two younger sisters, both having regular menstrual cycles. The elder of the two sisters has a healthy son. The mother is healthy, having one sister only who died in labour. The cause of death is unknown. Her father has epilepsy and has 5 healthy sibs (Fig. 1).

[1] Department of Obstetrics and Gynaecology, Postgraduate Medical School, and [2] Institute of Physical Education and Sport, Budapest, Hungary

Send offprint requests to J. László, H-1389 Budapest, Szabolcs u. 35, Hungary

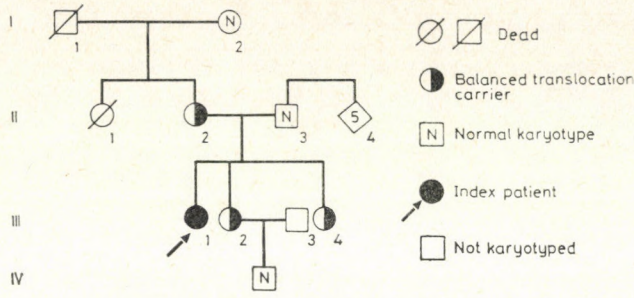



*Fig. 1.* Pedigree of the family

At the age of 15, the proband was of short stature (height 127 cm) with lack of breast development, infantile genitalia and a complete absence of pubic and axillary hair (Fig. 2). Her weight was 31.8 kg, with an arm span of 125.5 cm and 70.1 cm long lower extremities. She had brachycephaly, blue sclera, ptosis, short broad neck with low posterior hairline, high-arched palatale, abnormal dentition, shield chest, cubitus valgus, genu varum, coxa valga, irregularities of the epiphyses, hypoplasia of the frontal sinuses, and of the first cervical vertebra, bowed radius, Schmorl hernias, occult spina bifida, retarded ossification, decreased neurosensory hearing, hypoplastic superficial lymph vessels of the lower extremities as revealed by lymphangiography. Intravenous pyelography and echocardiography showed no anomaly. There was no colour blindness. Blood pressure was normal. Laparotomy revealed a rudimentary uterus, bilateral infantile Fallopian tubes and streak gonads which consisted of fibrous tissue without germ cells, follicles, or their remnants. At the age of 23 years, after 8 years hormonal replacement therapy, her height was 135.2 cm, weight 44.6 kg, arm span 135 cm and the length

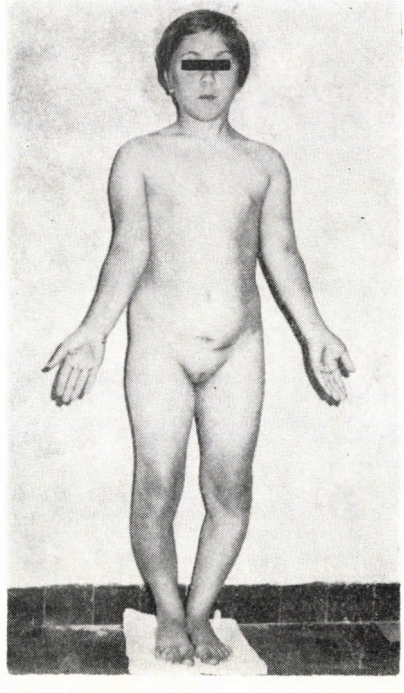


*Fig. 2.* Proband at age of 15 years

of the lower extremities 73.4 cm. Her intelligence appeared to be normal, with a verbal IQ of 98 and a performance IQ of 80 *(Wechsel Adult Intelligence Scale)*.

Thyroid function was normal. She had high serum LH and FSH levels (125 mU/ml and 140 mU/ml, respectively), normal levels of prolactin (12 ng/ml), and cortisol (180 nmol/l). The vaginal smear showed atrophy (maturation index 100/0/0). Urinary oestrogen excretion was low (3.7 nmol/d). Oral and intravenous glucose tolerance tests were normal. The patient and both of her parents were Xg/a/+.

The dermatoglyphic findings are shown in Table I. Fingertip patterns of the mother (II-2) and the youngest sister (III-4) were similar, with normal TRC. Increased frequency of whorls was observed in the proband (III-1) and in her first sister (III-2) with increased TRC and ARC. The father's (II-3) ridge count was not determined because of a wounded second finger on his right hand. Main-line terminations, especially of main-line C, were unusual in the proband and in her youngest sister (III-4), whereas complex palm pattern constellation and multiple secondary creases were observed in the proband and her mother. The proband had an incomplete simian crease on the right and a complete one on the left hand. Maximum atd angle of the mother and the proband's younger sister (III-4) was increased.

**Table I**

*Dermatoglyphic findings*

| | Fingertip patterns | | Maximum atd angle | TRC | ARC | Other |
|---|---|---|---|---|---|---|
| | Right | Left | | | | |
| Proband III 1 | $W^{dl}.W^{dl}.U^{lpv}.W^{ell}.U.$ | $W^{dl}.W^{dl}.W^{dl}.U^{lpv}.U.$ | 42° | 189 | 265 | Simian crease multiple secondary creases |
| II 2 | W.R.U.U.U. | U.U.U.U.U. | 76° | 145 | 158 | Multiple secondary creases |
| II 3 | $W^{ell}.?.W.W^{k}.U.$ | $U^{cpv}.U.U.U.U.$ | 47° | ? | ? | — |
| III 2 | $W^{dl}U^{cpv}U.W^{k}.U^{cpv}$ | $W^{dl}U.W^{k}.W^{ell}U^{cpv}.$ | 51° | 178 | 242 | — |
| III 4 | $W^{dl}.R.U.U.U.$ | U.R.U.U.U. | 68° | 150 | 170 | — |

*Palmar formulae**

| | Right | Left |
|---|---|---|
| Proband III 1 | 11.X.7.5'-t-$A^{u}$.O.O.O.O. | 9.X.5'-t-$A^{u}$.O.O.O.O. |
| II 2 | 11.9.7.5'-tt'$t^{b}$-W.O.O.L.O. | 9.9.5".5'-tt'$t^{b}$-W.L/V.O.1.O. |
| II 3 | 11.7.7.5'-t'-A"/$A^{c}$.O.O.L.O. | 9.7.5".5.-t-$A^{u}$.O.O.O.L. |
| III 2 | 9.7.5".5'-t't$^{b}$-$L^{carp}$ O.O.O.L. | 7.5".5".5'-t-$A^{u}$.O.O.O.L. |
| III 4 | 9.X.5".5'-t't$^{b}$-$L^{carp}$O.O.O.$V^{D}$ | 9.X.5'.5"-tt't$^{b}$-$L^{rad}/L^{uln}$O.O.O.$V^{D}$ |

* Main line terminations D.C.B.A.-axial triradius position-pattern areas (Hy.Th/ $I_1$. $I_2$. $I_3$. $I_4$.)

*Cytogenetic studies*

The X-chromatin was negative in the buccal smear. Cytogenetic studies were carried out according to our standard methods [2].

The patient had only one X chromosome and a monocentric 13/14 translocation chromosome (karyotype: 44,X, t [13; 14] [13qter → cen → 14qter)] (Fig. 3). The mother's and both sister's karyotypes were 45,XX, t (13; 14) (13qter → cen → 14qter). The father, the maternal grandmother (I-2) and the son of the elder sister of the proband (IV-1) had normal chromosome pattern.

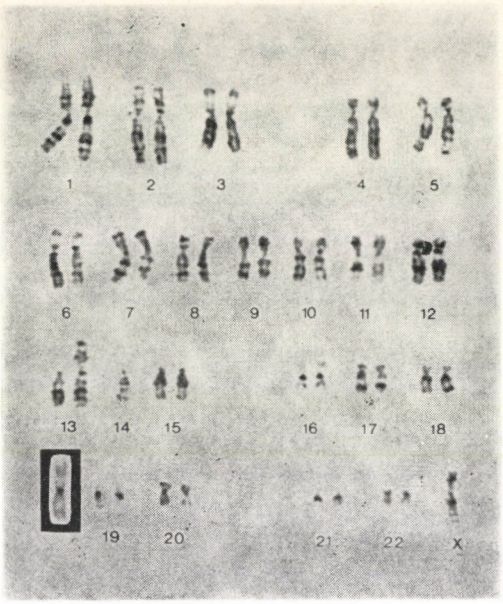



*Fig. 3.* G-banded chromosomes of the proband with t [13q14q] and X monosomy. Insertion shows monocentric translocation after C banding

## Discussion

Double major chromosome abnormalities such as double aneuploidy, or trisomy with structural rearrangements of unrelated chromosomes are infrequent in humans. The combination of X monosomy and translocations not involving the X chromosome even less usual. The present case is to our best knowledge, the second reported example of a streak gonad syndrome with X monosomy and simultaneous autosomal translocation. In the report of Kondo et al. [6] the X monosomy was combined with reciprocal translocation while in the present one with Robertsonian translocation.

The existence of an interchromosomal effect, i.e. the influence that a structurally abnormal chromosome may exert on the behaviour of other chromosomes, in humans is not known. A causal relationship between inherited balanced translocation and unrelated trisomy has been suggested [4, 10]. On the basis of Grell's [3] "distributive pairing" hypothesis Oikawa et al. [8] have suggested that as a result of distributive nondisjunction, the parental reciprocal translocation may be responsible for the unrelated trisomy. According to Kajii and Ferrier [5], the presence of a balanced translocation may influence segregation of other chromosomes at meiosis to cause nondisjunction. Other authors, however, questioned the suggested relationship between the two chromosome abnormalities [7, 9].

Whether the combination of X monosomy and familial balanced translocation involving autosomes is the result of chance or whether they are causally related is not known. Kondo et al. [6] comparing the occurrence and the expected frequency of the 45,X syndrome combined with unrelated balanced translocation postulated that there seemed to be no relationship between the two chromosome anomalies. In the present case there was no convincing evidence to indicate an interchromosomal effect, although the possibility cannot be excluded.

## Acknowledgements

We are indebted to Dr. É. Susa for help in dermatoglyphic studies, and Dr. Zs. Váczy for Xg blood group typing.

## REFERENCES

1. Bősze, P., László, J.: The streak gonad syndrome. Obstet. and Gynec. **54,** 544–548 (1979)
2. Gaál, M., László, J., Bősze, P.: Cytogenetic investigation of six patients with X isochromosomes, i(Xq), and of two subjects with isodicentric X chromosome, idic (Xq). Hum. Genet. **58,** 362–365 (1981)
3. Grell, R. F.: Distribution pairing in man? Ann. Genet. **14,** 165–171 (1971)
4. Hamerton, J. L., Gianelli, F., Carter, C. O.: A family showing translocation of a D/D reciprocal translocation and a case of regular 21 trisomy Down's syndrome. Cytogenetics **2,** 194–207 (1963)
5. Kajii, T., Ferrier, A.: Cytogenetics of aborters and abortuses. Amer. J. Obstet. Gynec. **131,** 33–38 (1978)
6. Kondo, I., Hamaguchi, H., Matsura, A., Nakajima, H., Koyama, A., Takita, H.: A case of Turner's syndrome with familial balanced translocation t(1; 2) (q32; q21) mat. J. med. Genet. **16,** 321–323 (1979)
7. Lindsteen, C., Vestermark, S., Philip, J.: A familial, balanced 2/5 translocation associated with trisomy 21 in one individual. Hum. Hered. **24,** 88–89 (1974)
8. Oikawa, K., Trent, M., Lebowitz, R.: Familial balanced translocation 4q+/17q- as a suggested cause of primary trisomy-21 Down's syndrome. Arch. Dis. Childh. **52,** 890–893 (1977)
9. Sparagana, M., Smith, G. F.: Klinefelter's syndrome associated with a D/D translocation. J. med. Genet. **12,** 299–301 (1975)
10. Subrt, I.: Reciprocal translocation with special reference to reproductive failure. Hum. Genet. **55,** 303–307 (1980)

*Acta Medica Hungarica, 41 (4), pp. 229–238 (1984)*

*Psychiatry*

# COMPARATIVE GENETICAL ANALYSIS: A NEW TOOL FOR VALIDATING THE SCHIZOPHRENIA SUBTYPES

G. UNGVÁRI

DEPARTMENT OF PSYCHIATRY, SEMMELWEIS UNIVERSITY MEDICAL SCHOOL, BUDAPEST, HUNGARY

(Received June 22, 1983)

350 carefully selected schizophrenic probands and their parents and siblings were diagnosed according to three different clinical classifications, and a multiple-threshold analysis was carried out within the framework of the multifactoral model of inheritance. The results suggest that of the three classification systems, Leonhard's and Sneshnevsky's nosological system delineates relatively homogeneous subtypes from the clinical and genetic point of view, pointing to the promise these nosological systems have for the planning of future research in psychiatric genetics.

**Keywords:** schizophrenia classification, genetics, multiple threshold strategy

## Introduction

Many of those accepted as authorities in psychiatric genetics are inclined to argue that only the discovery of unambiguous biological (pathogenetical) markers will signal an unambiguous advance in the study of the genetic and nosological problems of schizophrenia since the clinical and possibly genetic heterogeneity of schizophrenia poses insolvable problems to phenomenological research [3, 7, 10]. Committed nosologists on the other hand are unanimous in their claim that only precisely formulated psychopathological diagnoses, or rather their coherent system, i.e. a nosology will make it possible to establish the biological indicators. Both these views appear justified at first sight, thus forming a vicious circle. The merely apparent nature of the latter however becomes obvious if we bear in mind that skepticism vis a vis the phenomenological approach is predominantly based on disillusionment with nosology of the Kraepelin–Bleuler sort and its computerized neo-Kraepelin versions [14]. It should be added that though psychiatric genetics is more than sixty years

Send offprint requests to G. Ungvári, H-1083 Budapest, Balassa u. 6, Hungary

old, its methodology has only become crystallized in the past decade and no clinical-genetical study has satisfied all the criteria of this methodology, at the most they have come close to doing so. Lacking an appropriate methodology it was only natural that the mathematical and statistical methods of modern quantitative genetics were seldom used [1]. It can therefore be said, and many geneticists who are not committed to nosology also stress this [4], that the phenomenological approach to the genetical investigation of schizophrenic psychoses can certainly not be said to have had its day. It is indeed still timely. For a number of reasons clinical diagnosis as the starting point of research and its reference system, as the most crucial methodological problem, is with no doubt still the most critical area of psychiatric genetics.

1. The subjectivity of diagnosis is the least of the problems since its effects can easily be eliminated by formalization of the psychiatric interview and description, and the measurement and raising of the level of reliability.

2. More difficult to eliminate are the radical differences of approach of various nosologies, making it impossible to compare results obtained by different schools. It is clear that the WHO diagnostic schemata [19] facilitate communication but do not offer a solution. In my own view rather than employing exclusively ICD-9 or any other diagnostic schemata, it would be more suitable if, even in theoretic work, psychiatric genetics were to chart the link-up and overlap of a number of nosologies making the results obtained suitable for comparison in this manner. Recently this strategy has been recommended for biological psychiatry [7].

3. The third major factor in the incommensurability of results is that each of the surveys with a psychiatric genetics purpose was carried out on different populations. Among the factors that remain unknown are population gene-pool differences and numerous other socio-cultural factors that do not influence schizophrenia as such but the conditions of its recognition and identification, such as the tolerance shown to mental disoders by a given society, the incidence and changing frequency of inbreeding and assortative mating. And yet, as Kety [6] notes, sociocultural factors are unjustifiably taken as constant.

The present paper reports on an attempt to eliminate as far as possible such methodological shortcomings in genetic research of schizophrenia, inasmuch as they are linked to diagnosis, by concurrently diagnosing identical probands and their families according to three separate classifications. The aim was to attempt, by surveying the same patient-population and employing strict methodological criteria, to discover in as precise a way as possible which of the classifications used offers sub-groups that are most homogeneous from the clinical and genetic point of view, and thus most suitable for further genetical and biological research. The results of the present clinical-genetical survey serve as one of the validity criteria of these classifications [13].

Of the three classifications two, that by Leonhard [8] and that of Sneshnevsky [16] are amongst the most detailed and most systematic, well-elaborated schemes based in part, on clinical-genetic foundations. The Leonhardian nosology [8], as the development of the Kleist–Wernicke school of classical German psychiatry, is strictly empirical, meticulous, and characterized by systematic and detailed psychopathological observations. Within the two basic categories of the schizophrenic psychoses (systematic and unsystematic), 19 subtypes are described as distinct disease entities, and further 3 subtypes of cycloid psychosis are delineated out of the group of functional psychoses. Cycloid psychoses closely resemble the schizoaffective psychoses (according to ICD-9) or the recurrent schizophrenic psychoses (in Sneshnevsky's classification).

In Sneshnevsky's classification, widely used in the Soviet Union, refined details of cross-sectional psychopathology have less importance. The system classifies the schizophrenic psychoses by the course of illness and by the degree of severity in the deterioration of mental functions. According to this descriptive method, there are 3 basic types of schizophrenia (continuous, shift-like, recurrent) and every type has further 3 subtypes, for details, see [16].

ICD-9, the official classification of WHO [19], was included as the model of the traditional Kraepelin–Bleuler classification of schizophrenias, based on the clinically most frequent formes. This is, because of its practical usefulness and relative simplicity, a widely used classification mainly in Europe, but also throughout the world.

## Subjects and method

The methodology was discussed in detail in earlier papers [17, 18] and therefore its brief summary will only be given. The schizophrenic probands and their first degree relatives were diagnosed on the basis of detailed case records constructed according to special genetic considerations. The case records were chosen bearing in mind the points listed below from those kept at the Genealogical Archives of the Institute of Psychiatry, Moscow.

The Archives are a representative sample of the Moscow schizophrenic population [5]. Strict methodological criteria were employed in an endeavour to reduce to minimum the deficiencies of the common chart review method of psychiatric genetics. The Genealogical Archives include the medical documentation of 700 schizophrenic probands and their families. Of these, all those case records were studied where (*i*) detailed medical documentation was available for at least five years following the first hospitalization; (*ii*) the proband had reached 18 years by the time of the last follow up; and (*iii*) the first hospitalization took place before the age of sixty. In this way both childhood schizophrenia and schizophrenia in the elderly were excluded. Three hundred and fifty probands satisfied these initial conditions.

A diagnosis was separately formulated for all 350 patients and for each of the three clinical systems, rigorously adhering to the preformed and pre-tested diagnostic schemata, thus reducing the distorting effects of subjectivity, and ensuring that the examination could be controlled and repeated. According to the Kraepelin–Bleuler classification, diagnoses were established using the WHO official diagnostic schemata and guidelines [19]. For the other two nosological systems two sets of diagnostic criteria were compiled on the basis of current handbooks [8, 16] and under the personal control of Sneshnevsky and his associates and of Leonhard. In the two diagnostic schemata the original descriptions were transferred into a criteria set to operationalize the diagnostic process. In both diagnostic schemata a given

basic schizophrenia type is characterized by 3–5 main criteria, a subtype within this basic type is defined by further 6–12 additional criteria. A firm diagnosis was established if a patient's disease had met all the main criteria and at least 75% of the additional criteria. (The two sets of criteria for diagnosing Leonhard's and Sneshnevsky's system are available from the author).

Diagnosis was made without knowledge of the original institutional diagnosis of the patients, the psychopathology of their relatives or the nature of the relationship, since the relevant information had been removed from the case-records. Cases which could not be diagnosed reliably, and those doubtful according to all three systems of classification, were eliminated since the aim was a homogeneous sample. Since the patients were not examined personally, borderline psychoses and personality disorders have not been evaluated. The smallness of the sample implied that only basic schizophrenia subtypes of the particular classifications could be examined. Some of the characteristics of those unambiguously diagnosed as schizophrenics are listed in Table I separately for each of the three systems.

**Table I**

*Some basic characteristics of the probands according to the three diagnostic systems*

| Diagnostic system | Nosological diagnosis of schizophrenia | Age of onset of psychosis, years | Duration of follow up periods, years | Estimated population (lifetime) prevalence, percent |
|---|---|---|---|---|
| ICD-9 | simple (N = 18) | 17.0±3.0* | 12.4±7.9 | 0.064 |
| | hebephrenic (N = 27) | 17.1±2.6 | 11.6±5.8 | |
| | catatonic (N = 39) | 17.4±3.0 | 15.6±8.2 | 0.085 |
| | paranoid (N = 84) | 23.7±6.9 | 11.4±4.1 | 0.597 |
| | schizoaffective (N = 67) | 26.6±9.1 | 14.8±8.5 | 0.195 |
| Leonhard | systematic (N = 117) | 19.6±6.4 | 12.4±5.4 | 0.335 |
| | non systematic (N = 71) | 20.0±5.2 | 12.4±6.8 | 0.247 |
| | cycloid psychosis (N = 68) | 26.2±8.9 | 15.0±8.8 | 0.342 |
| | "continuous" (N = 96) | 18.6±4.5 | 13.1±6.1 | 0.190 |
| Sneshnevsky | "shift-like" (N = 132) | 21.3±7.0 | 11.9±5.4 | 0.604 |
| | "recurrent" (N = 61) | 25.9±8.4 | 15.2±9.1 | 0.195 |

*mean ± S. D.
(N = number of probands)

The children of probands were not included in view of their small number and their age. The case records of the parents and siblings of probands were evaluated regardless of their age or the duration of the follow-up period. Relatives were evaluated in the same way as probands, without knowledge of the institutional diagnosis or the nature of the relationship. I had access to the documentation of 91% of all the parents and siblings.

**Table II**

*Primary data for threshold analysis of basic schizophrenia subtypes classified according to ICD-9*

| Schizophrenia subtypes according to ICD-9 | No. of probands | Parents and siblings | | | | |
|---|---|---|---|---|---|---|
| | | Total | With schizophrenic psychoses | | | |
| | | | S | C | P | SA |
| Simple (S) | 18 | 55 | 2 | — | — | — |
| Catatonic (C) | 39 | 132 | — | 3 | 1 | — |
| Paranoid (P) | 84 | 290 | 1 | 1 | 10 | 2 |
| Schizoaffective (SA) | 67 | 257 | 1 | — | 3 | 4 |

The morbidity risks among relatives of schizophrenic psychoses have been discussed elsewhere [17, 18]. It suffices to mention that the risk of schizophrenia of the relatives according to the classifications agreed in their trend with the views of accepted authorities. This at the same time tends to confirm that the compiled and employed diagnostic schemata and the diagnoses themselves, at least came close to the spirit and prescriptions of the three separate nosologies.

The empirical (so-called primary) data for schizophrenia amongst relatives (Tables II–IV) were subjected to multiple-threshold analysis and to genetic heterogeneity test within the framework of the multifactoral model of inheritance [11, 12, 15]. These tests are suitable for deciding on the adequacy of a given classification [12]. The essence of the multiple-threshold strategy is the hypothesis that the liability for a given disease, manifesting clinically with various types and subtypes, shows normal distribution in the whole population as well as in the relatives of the probands. In this respect liability means the simultaneous presence of genetical and environmental factors which lead to the appearence of the given disease. The various types (and subtypes, if they exist) manifest themselves at various threshold of the normally distributed liability curve. According to the multiple-threshold method, one of the two types of the disease studied is judged to be the "narrow" form, differing genotypically more from the population average and being clinically more severe than other types, called "wide" form in this context. This is the basic principle of the mathematical-statistical computations (for details see Refs [1, 11, 12]).

**Table III**

*Primary data for threshold analysis of schizophrenia subtypes and cycloid psychoses classified according to Leonhard's system*

| Nosological diagnoses | No. of probands | Parents and sibs | | | |
|---|---|---|---|---|---|
| | | Total | With schizophrenic psychoses | | |
| | | | SSY | NSSY | CP |
| Systematic schizophrenia (SSY) | 117 | 405 | 9 | — | — |
| Non-systematic schizophrenia (NSSY) | 71 | 244 | 5 | 10 | 3 |
| Cycloid psychoses (CP) | 68 | 263 | 1 | 3 | 8 |

**Table IV**

*Primary data for threshold analysis of schizophrenia subtypes classified according to Sneshnevsky's system*

| Schizophrenia subtypes | No. of probands | Parents and sibs | | | |
|---|---|---|---|---|---|
| | | Total | With schizophrenic psychoses | | |
| | | | CS | SS | RS |
| Continuous (CS) | 96 | 311 | 7 | 2 | — |
| Shift-like (SS) | 132 | 454 | 3 | 31 | 1 |
| Recurrent (RS) | 61 | 237 | — | 2 | 10 |

Multiple-threshold analysis has become increasingly popular in psychiatric research to test the presumed mode of inheritance of mental diseases [1, 2]. The starting point of the present study is slightly different. In this paper the most likely multifactoral model of inheritance was taken as given on the basis of the morbidity risks, attention being concentrated on the interrelationship of the subtypes of schizophrenic psychoses, within a given nosological system, i.e. testing the adequacy of the three classifications.

The computer programme was written on the basis of the formulations of Reich et al. [11] and Smith [15] in the Institute of Psychiatry, Moscow, and processed on a WANG-2200 computer. Surveys carried out by the Department of Epidemiology of the same Institute

supplied the population prevalence of particular subtypes. As regards the Leonhard system, the appropriate data were considered on the basis of Leonhard's Berlin surveys (Leonhard, personal communication, 1981). The heritability ($h^2$) values of the subtypes were computed by the usual Falconer method (for details, see Refs [11, 12]).

**Table V**

*Results of multiple-threshold analysis and test of genetic heterogeneity of some subtypes of schizophrenia according to ICD-9*

| ICD-9 schizophrenia subtypes (I) | ICD-9 schizophrenia subtypes (II) | | | |
|---|---|---|---|---|
| | S | C | P | SA |
| Simple | $h^2 = 0.93$ | $x^2(I) = 4.7$<br>$x^2(II) = 8.4$ | $x^2(I) = 6.3$<br>$x^2(II) = 26.0$ | $x^2(I) = 4.2$<br>$x^2(II) = 18.4$ |
| Catatonic (C) | $r_g = 0$ | $h^2 = 0.75$ | $x^2(I) = 5.1$<br>$x^2(II) = 15.7$ | $x^2(I) = 6.1$<br>$x^2(II) = 15.3$ |
| Paranoid (P) | $r_g = 0.35$ | $r_g = 0.25$ | $h^2 = 0.53$ | $x^2(I) = 5.2$<br>$x^2(II) = 6.6$ |
| Schizoaffective (SA) | $r_g = 0.47$ | $r_g = 0$ | $r_g = 0.41$ | $h^2 = 0.50$ |

$h^2$ = heritability; $r_g$ = genetical correlation coefficient;
($r_g$ does not differ significantly from zero in any case at the 5% level)
Controlling the hypothesis of the same liability continuum for two subtypes (i.e. $r_g = 1$ between them), using $2 \times 2$ contingency tables, the critical value of $x^2 = 11.3$ ($p < 0.01$)
I = values of $x^2$ for subtypes listed vertically and II = values of $x^2$ for subtypes listed horizontally, as for the "narrow" form of the disease.

## Results

Tables VI–VII sum up the computation carried out for the three clinical systems. The Tables contain not only the results of multiple-threshold analyses ($x^2$/I and $x^2$/II) but also the heritability ($h^2$) values of the subtypes as well Smith's correlation coefficients ($r_g$) expressing the degree of genetic heterogeneity.

**Table VI**

*Results of multiple-threshold analysis and test of genetic heterogeneity referring to systematic and non-systematic schizophrenias and cycloid psychoses*

| Nosological diagnosis according to Leonhard (I) | Nosological diagnosis according to Leonhard (II) | | |
|---|---|---|---|
| | SSY | NSSY | CP |
| Systematic schizophrenia (SSY) | $h^2 = 0.50$ | $x^2(I) = 35.9$<br>$x^2(II) = 11.8$ | $x^2(I) = 16.5$<br>$x^2(II) = 17.9$ |
| Non-systematic schizophrenia (NSSY) | $r_g = 0.53^*$ | $h^2 = 0.77$ | $x^2(I) = 4.3$<br>$x^2(II) = 24.2$ |
| Cycloid psychoses (CP) | $r_g = 0.02$ | $r_g = 0.50^*$ | $h^2 = 0.60$ |

* Statistically significant differences from zero at the 5% level.
For explanations, see Table V

**Table VII**

*Results of multiple-threshold analysis and test of genetic heterogeneity of basic subtypes of schizophrenic psychoses according to Sneshnevsky's system*

| Nosological diagnosis according to Sneshnevsky (I) | Nosological diagnosis according to Sneshnevsky (II) | | |
|---|---|---|---|
| | CS | SS | RS |
| Continuous schizophrenias (CS) | $h^2 = 0.61$ | $x^2(I) = 40.9$<br>$x^2(II) = 17.2$ | $x^2(I) = 30.8$<br>$x^2(II) = 14.4$ |
| Shift-like schizophrenias (SS) | $r_g = 0.23$ | $h^2 = 0.80$ | $x^2(I) = 31.5$<br>$x^2(II) = 31.3$ |
| Recurrent schizophrenias (RS) | $r_g = 0$ | $r_g = 0.07$ | $h^2 = 0.82$ |

For explanations see Table V

## Discussion

Owing to the ten schizophrenia subtypes of ICD-9, few patients could be classified in the traditional subtypes (simple, hebephrenic, catatonic, paranoid) that were of particular interest. As a result, hebephrenia was in fact left out of the analysis. As Table V shows the heritability of liability for psychosis ($h^2$), it is closely paralleled by an unfavourable clinical outcome and resistance to therapy. The validity of the heritability figures obtained is borne out by clinical experience, which shows that psychosocial factors have a great role in the schizoaffective and paranoid schizophrenias ($h^2 = 50$ and 53%, respectively). Bearing in mind the smallness of the sample, from the $r_g$ values it may be presumed that the genes responsible for the psychosis of patients of a given subtype are identical only to a small extent. The multiple-threshold model refutes the possibility of a specific gene-constellation of the ICD-9 schizophrenia subtypes. The hypothesis that liability was the same, was confirmed for every case ($x^2/I < 11.3$ in every case). Thus, according to the multiple-threshold model every subtype finds its place at various points of the same liability-continuum, and their manifestations occur at different thresholds. The simple subtype of the highest heritability is the most "narrow" form: its presumed genetic constellation is more likely to differ from the population average, while the schizoaffective subtype of low heritability, the "broadest" disease-form, differs least in gene-structure from the population average. In keeping with the demands of the model, the outcome is also clinically the most favourable in the latter case.

To sum up, the two models (those of Reich et al. and Smith) based on phenotypical correlation have produced contradictory results inasmuch as the existence of a specific and independent genotype for each subtype was not unambiguously confirmed. Two possible explanations are suggested: (*i*)

the clinical-psychopathological principles of the ICD-9 are adequate, but genetical factors have no role, or only a small one, in defining the subtypes; (*ii*) one can differentiate genotypically between various subtypes within the spectrum of schizophrenic psychoses but the ICD-9 clinical classification principles are inadequate, that is they do not differentiate on the basis of genetic differences but according to other psychopathological phenomena that lack a genetical link. Bearing in mind that there are no unambiguous biological indicators (pathogenetic markers) for schizophrenia, one can only escape the dilemma at the phenomenological level, through an analysis of alternative clinical systems using an identical methodology. It is precisely this that supplies the methodological advantages of the present comparative study. Identical genetical analytical methods are used on an identical patient population, thus making the various nosological systems, of schizophrenia comparable, in principle without distortion, at least from the genetical point of view. I am not aware of any reported similar research in the psychiatric genetic literature accessible to me.

Analysis of only the basic subtypes of the Leonhard classification of schizophrenias and cycloid (schizoaffective) psychoses is reported here (Table VI). In keeping with the expectations of the Leonhard system, the heritability of the non-systematic subtype is the highest while that of systematic schizophrenies is only 50%. In the genesis of the latter Leonhard attributes an outstanding role to psychosocial factors. The $r_g$ unambiguously indicates the genotypical distinctness of cycloid psychoses and systematic schizophrenias. The non-systematic group displays a considerable genotypical overlap with both other groups, as it were a connecting link. Multiple-threshold analysis, rejecting the possibility of identical liability, confirmed the separateness of cycloid psychoses and systematic schizophrenias ($x^2$[I] and [II] = 16.5 and 17.9, respectively). There is no shared liability of systematic and non-systematic schizophrenia. It appears clinical criteria are least able to differentiate between non-systematic forms and cycloid psychoses, $r_g$ between them is high and the hypothesis of identical liability is verified ($x^2$[I] = 4.3 < 11.3). Thus, the clinical classification of schizophrenias and genetical classifications are closer to each other on the basis of Leonhard's nosological system than was the case for ICD-9.

Sneshnevsky's is the third classification subjected here to comparison. Table VII shows that the three basic subtypes of schizophrenia coincide in their separateness according to both models, the clinical grouping is presumably backed by subforms that differ genetically, and that are more homogeneous than those discussed above.

The relatively small sample is a shortcomming of the present investigation, the results are therefore of a preliminary nature; for the time being they only indicate trends. The clinical-genetic validation of the classification of

schizophrenias employing the methods of quantitative genetics thus suggests that (*i*) the course of the illness and (*ii*) the degree of severity of personality deterioration, that is the two basic classificatory criteria of Sneshnevsky's system produce subtypes that are more homogeneous genetically than Leonhard's system based on scrupulous psychopathological observation or the traditional cross-sectional (syndromatological) grouping of the ICD-9 classification.

Although their classification principles are different, the psychopathological similarity and overlap between the Leonhardian and Sneshnevskyan nosologies are striking. This fact explains the similar results of genetic analysis.

The present results are only preliminary, yet they give hope that, given a broadening of the research strategy, they will inspire further investigations. In the long run, biological variables that can be used as potential genetic markers (dermatoglypha, blood groups, nailfold capillary pattern, MAO enzymes, etc.) and greater precision in psychopathological analysis appear to be essential.

## Acknowledgements

The author is indebted to Prof. K. Leonhard, Prof. A. Sneshnevsky, Prof. M. E. Vartanian, Prof. I. V. Schachmatova and V. M. Gindilis for their helpful assistance in preparing this paper though they are of course in no way responsible for its shortcomings.

## REFERENCES

1. Baron, M.: Genetic models of schizophrenia. Acta Psychiat. Scand. **65,** 263–275 (1982)
2. Baron, M., Gruen, R., Asnis, L., Kane, J.: Schizoaffective illness, schizophrenia and affective disorders: morbidity risk and genetic transmission. Acta Psychiat. Scand. **65,** 253–262 (1982)
3. Canro, R.: Genetic evidence for the existence of subgroups of the schizophrenic syndrome. Schiz. Bull. **5,** 453–459 (1979).
4. Fuller, J. L., Thomson, W. R.: Foundation of Behavior Genetics. Mosby, St. Louis 1978
5. Gindilis, V. M.: The genetics of schizophrenic psychoses. D. Sc. thesis, Moscow 1979
6. Kety, S. S.: Problems of genetic research on psychiatric illness. In: Gershon, E. S., Matthysse, S., Breakfield, X. O., Ciaranello, R. B. (eds): Genetic Research Strategies in Psychobiology and Psychiatry. Elsevier, Amsterdam 1981, pp. 395–397.
7. Kendell, R. E.: The choice of diagnostic criteria for biological research. Arch Gen. Psychiat. **39,** 1334–1339 (1982)
8. Leonhard, K.: The Classification of Endogenous Psychoses. Irvington, New York 1979.
9. Mitsuda, H.: Clinico-genetic studies of schizophrenia. In: Mitsuda, H. (ed.): Clinical Genetics in Psychiatry. Bunko-Sha, Kyoto 1967, pp. 49–91.
10. Odegard, O.: Comments of the genetic issue. Schiz. Bull. **3,** 345–347 (1977)
11. Reich, T., James, J. W., Morris, C. A.: The use of multiple threshold in determining the mode of transmission of semicontinuous traits. Ann. Hum. Genet. **36,** 163–184 (1972)
12. Reich, T., Cloninger, C. R., Guze, S. B.: The multifactorial model of disease transmission. I. Description of the model and its use in psychiatry. Br. J. Psychiat. **127,** 1–10 (1975)
13. Robins, E., Guze, S. B.: Establishment of diagnostic validity in psychiatric illness: its application to schizophrenia. Am. J. Psychiat. **126,** 983–987 (1970)
14. Shepherd, M.: Definition, classification and nomenclature: a clinical overview. In: Kemali, D., Bartholini, G., Richter, D. (eds): Schizophrenia Today. Pergamon, Oxford 1976, pp. 3–12.

15. Smith, Ch.: Statistical resolution of genetic heterogeneity in familial disease. Ann. Hum. Genet. **39**, 281–291 (1976)
16. Sneshnevsky, A. V.: Symptomatology and nosology. In: Sneshnevsky, A. V. (ed.): Schizophrenia. Medicine, Moscow 1969, pp. 5–29.
17. Ungvári, G.: An attempt to explore the validity of Leonhard's classification of endogenous psychoses. Arch. Psychiat. 1983a (in press)
18. Ungvári, G.: On the validity of the ICD-9 schizophrenia classification. Acta Psychiat. Scand. 1983b (in press)
19. WHO: Mental Disorders: Glossary and Guide to Their Classification in Accordance with the 9th Revision of the ICD. WHO, Geneva 1978.

*Acta Medica Hungarica, 41 (4), pp. 239—246 (1984)*

*Hepatology*

# NON-SPECIFIC DRUG-METABOLIZING ENZYME ACTIVITY IN GILBERT DISEASE

Ágnes VÁRKONYI, Teréz SZELECZKI, Jolán CSANÁDI, K. GYURKOVITS

DEPARTMENT OF PAEDIATRICS AND INSTITUTE OF PATHOLOGY, UNIVERSITY MEDICAL SCHOOL, SZEGED, HUNGARY

(Received February 4, 1983)

Gilbert disease was diagnosed in a 15-year-old boy on the basis of the clinical pattern and the changing drug-metabolizing capacity of the liver-enzyme apparatus, which was drug-induced.

**Keywords:** Gilbert disease, antipyrine half-life, D-glucaric acid elimination.

## Introduction

According to the classical definition, chronic or recurrent, mild, non-conjugated hyperbilirubinaemia occurs in Gilbert disease (G.d.), but liver function and histology are normal, and there is no haemolysis. It has, however, been discovered that haemolysis and G.d. do not exclude each other; in some patients abnormal liver function and bilirubin clearance may occur together with hyperbilirubinaemia; even minor morphological anomalies such as accumulation of lipofuscin-like material can be observed and may even be characteristic [22, 23].

Many reports have shown that in G.d. the reduced activity of UDP-glucuronyl transferase was responsible for the symptoms. Measurement of the transferase activity alone cannot, however, be used as a diagnostic test, because reduced levels have been observed also in other conditions such as chronic persistent hepatitis, Wilson's disease, and haemolytic disease [2, 4].

Thus the diagnosis was based on the clinical picture, the liver biopsy, and when possible, determination of the liver bilirubin UDP-glucuronyl-transferase level.

We report here the case of a 15-year-old boy, in whom the diagnosis was confirmed by the changing drug-metabolizing capacity of the liver enzyme apparatus.

Send offprint requests to Ágnes Várkonyi, H-6701 Szeged, P.O. Box 471, Hungary

## Patient and methods

The 15-year-old brother of a 3-year-old boy now under gastroenterological treatment because of primary saccharose malabsorption since early infancy — was admitted for check-up. Two years before he had developed jaundice after a febrile illness without any previous treatment. Since then he has had recurrent episodes of jaundice with fatigability, general discomfort, mild diarrhoea, and headaches.

On admission the asthenic patient's height was 97%, his body weight 30%. Apart from icteric sclerae, no abnormality was found on physical examination. The liver and spleen were not enlarged.

For confirmation of the diagnosis antipyrine (18 mg/kg orally) half-life in saliva and the excretion of D-glucaric acid in urine were measured and these examinations were repeated after two weeks treatment with flumecine (10 mg/kg/day). (Zixoryn® G. Richter, Budapest.) The results are to be seen in Tables I and II.

**Table I**

*Antipyrine half-life in the saliva and D-glucaric acid elimination in the urine of the patient and his parents*

| | Antipyrine half-life h | D-glucaric acid elimination μM/g creatinine/24 h |
|---|---|---|
| Patient | 22.3 | 15.1 |
| Controls (n=18) | 7.86±0.74 | 33.43±5.54 |
| Mother | 23.2 | 33.6 |
| Father | 16.9 | 12.1 |
| Adult controls in literature Clin. Pharm. Ther. *17*, 179 (1976) | 13.58±0.76 | |

Values are mean ± S.E.M.

**Table II**

*Antipyrine half-life, D-glucaric acid, and serum bilirubin level of the patient before and after induction*

| | Before induction | After induction |
|---|---|---|
| Antipyrine half-life, h | 22.3 | 14.9 |
| D-glucaric acid elimination, μM/g creatinine/24 h | 15.1 | 60.0 |
| Serum bilirubin, μM/l, indirect | 76.9 | less than 17.0 |

At the same time we determined the serum bilirubin level and the GOT and GPT values. For weeks before, and the time of the examinations the patient was not receiving drug therapy.

In order to assess the therapeutic effect, 10 mg/kg flumecine was given once a week throughout 12 weeks. The above-mentioned parameters were measured 48 hours after administration of the drug, 8 times altogether. The results are summarized in Table III.

For measuring the concentration of D-glucaric acid in urine we used March's method [17] as modified by Simons et al. [28].

Antipyrine concentration was measured according to the method of Brodie et al. [16].

**Table III**

*Antipyrine half-life in the saliva and D-glucaric acid elimination in the urine of the patient during 12 weeks flumecine treatment*

| Study weeks | Antipyrine half-life h | D-glucaric acid elimination μM/g creatinine/24 h |
|---|---|---|
| 1 | 10.9 | 20.9 |
| 2 | 6.3 | 14.1 |
| 3 | 12.1 | 26.7 |
| 4 | 14.7 | 31.6 |
| 6 | 13.0 | 32.8 |
| 8 | 6.5 | 42.2 |
| 10 | 7.9 | 38.9 |
| 12 | 6.7 | 37.0 |

## Results

Blood counts: PCV 0.41, Hb 132 g/l, reticulocytes 0.009, WBC $5.4 \times 10^9$/l; band 0.01, se 0.42, ly 0.54, eo 0.03. Urine: protein, pus, sugar: negative, ubg: normal.

Serum bilirubin: 98; 67; 32.4; 71; 25.6 μmol/l indirect; GOT: 8 U/l, GPT: 9.6 U/l, alkaline phosphatase: 66 U/l, thymol: 0.4 TU, total protein: 68 g/l, albumin: 40.5 g/l, total lipid: 5.9 g/l, cholesterol: 5.2 mmol/l, triglyceride: 0.57 mmol/l, saccharose tolerance test: normal. (Oral tests proved that there was saccharose malabsorption in the child's mother and the mother's sister.)

Se iron: 26.7 μmol/l, TIBC: 58.1 μmol/l, IgA: 1.1 g/l, IgM: 1.7 g/l, IgG-6.93 g/l, coeruloplasmin: 59 U/l, direct Coombs: negative, indirect Coombs: negative, with several foreign and own cells, RBC osmotic fragility test: 0.46%–0.30% NaCl; RBC morphology: normal, ECG: normal, oral cholangiography: normal.

Light and electron microscopy of liver biopsy: intact lobular structure, normal arrangement of liver cells which are rich in glycogen (especially rosette forms). There is a considerable amount of nuclear glycogen consisting exclusively of monoparticular glycogen and diastase-resistant PAS-positive nuclear inclusions are seen. The mass of rough-surface endoplasmic reticulum decreased, that of smooth reticulum increased. There were sporadic giant mitochondria, occasionally with paracrystalline structures in them. In some liver cells there were numerous lipofuscin granules, many of them containing lipid drops.

On the vascular pole of the liver cells the cell membrane was flattened out, otherwise no change could be detected. In some liver cells small lipid drops could be observed, while the Kupffer cells showed advanced fatty degeneration. At some portal sites there was slight mesenchyma cell proliferation (Figs 1, 2, 3).

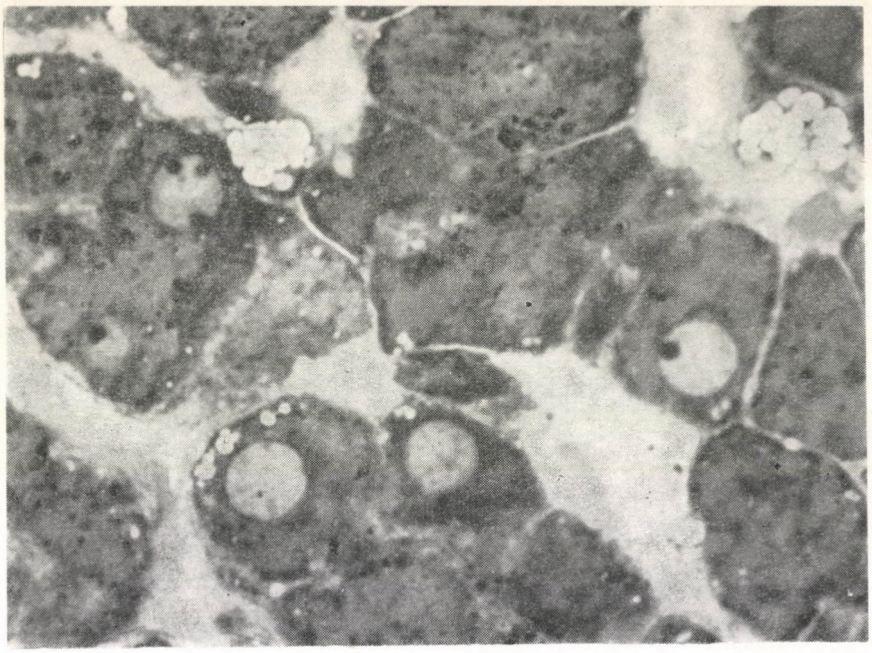


*Fig. 1.* Light microscopic picture of liver biopsy specimen. The hepatocytes contain especially near the bile ducts much lipofuscin (black granules). In some hepatocytes there are small fat vacuoles; two Kupffer cells are entirely filled with fat vacuoles. Half-thin section, methylene blue and basic fuchsin staining, ×1400

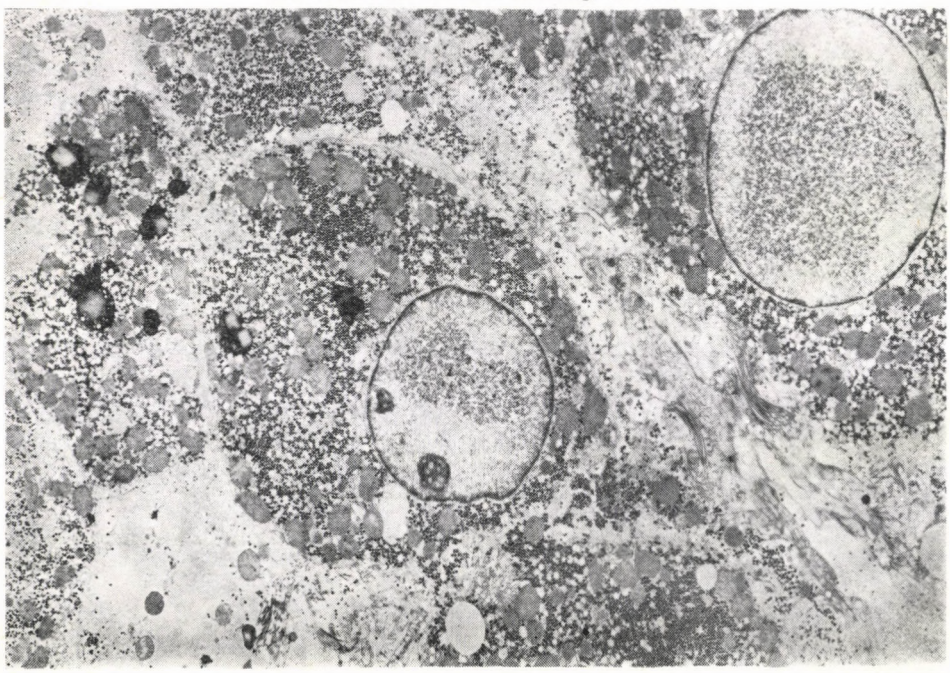


*Fig. 2.* Electron microscopic picture of liver biopsy specimen. In the cell nuclei nuclear glycogen. Among the mitochondria the cytoplasm of the hepatocytes contains much glycogen. ×5000

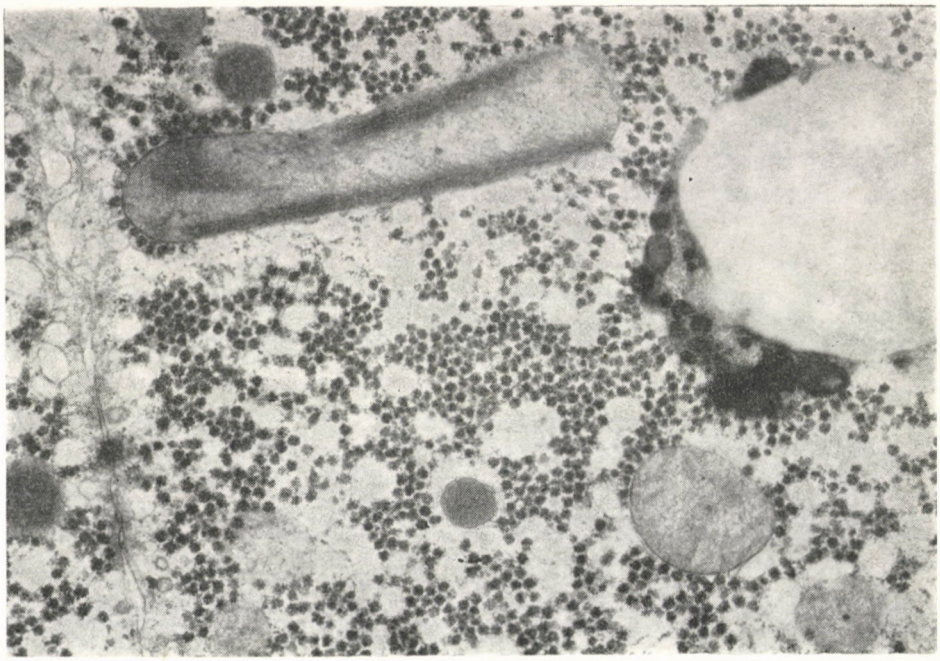


*Fig. 3.* Electron microscopic picture of liver biopsy specimen. Parts of two adjacent hepatocytes. The lipofuscin-like pigment contains a large lipid drop. There is paracrystalline formation in a giant mitochondrion. ×27 000

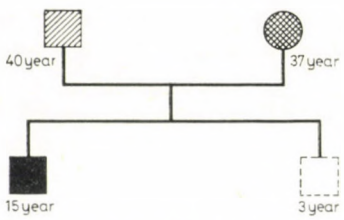



*Fig. 4.* Enzyme activity in the family examined

## Discussion

Gilbert and Lereboullet's paper published in 1901 (cit. [2]) provoked many discussions later when the metabolism of bilirubin had become fully known. The question was then raised whether the cases reported by these authors were really what is now called G.d. [26] or whether the disease was a separate entity [1].

The appearance of several papers [10] and a book [22] prove that the disease is none too rare, but it is not always recognized.

It may be misleading that the mild hyperbilirubinaemia is recognized often only after a febrile illness [26], after drug therapy [11], or in haemolytic

stress [22]; the picture is often complicated by systemic symptoms, which, according to some authors, are independent of the pigment disturbance and appear mostly in neurotic patients [26]. In our case, too, there were several systemic complaints, which however could not entirely be attributed to the patient's awareness of the disease.

In G.d. spontaneous fluctuations of non-conjugated hyperbilirubinaemia occur, which make the diagnosis difficult. The fluctuation is influenced by many factors, thus by physical exertion, emotional excitement, infections, and alcohol; recently even a reciprocal connection was found between calory intake and the serum bilirubin level [8]. In spite of the fluctuation the bilirubin level does not usually rise above 100 μmol/l. In our case fluctuations between normal values and 98 μmol/l were observed.

There is no specific method for the demonstration of G.d. We have confirmed the diagnosis by liver biopsy and by measuring the activity of non-specific drug-metabolizing enzymes [19, 20, 21].

It is known that changes in the liver tissue cannot always be observed by light microscopy. There is no sign of necrosis or biliary retention. Deposition of a lipofuscin-like substance [12] as in our case may be characteristic. A number of ultrastructural abnormalities have also been detected [18, 24], and with morphometric examinations even significant abnormalities have been found, but they are characteristic not only of G.d. [12].

In our case the morphological picture was nearly identical with that described in G.d. [7, 9, 13, 25, 27, 29]. The lipofuscin-like pigment, which is considered most characteristic, but which often contains lipids, hypertrophy of the smooth-surfaced endoplasmic reticulum and flattening of the vascular pole of the hepatocytes were clearly discernible as were also the mitochondrial changes. To our knowledge the morphological picture of G.d. does not include such changes as the very pronounced fatty degeneration of Kupffer cells in our case and the presence of a number of nuclear inclusions, which latter proved to be mostly monoparticular glycogen and in a small proportion diastase-resistant PAS-positive matter.

Table I shows the half-life of antipyrine and the values of D-glucaric acid excretion in the family members examined. Table II shows the changes of the above-mentioned parameters as a result of flumecine treatment, which caused a marked decrease in the serum bilirubin level.

The antipyrine half-life and D-glucaric acid values are seen in Table III. The data seem to support the therapeutic value of flumecine if it is considered feasible depending on the specific condition.

The liver tests were within normal limits during treatment.

D-glucaric acid is the end product of glucuronic acid metabolism, and thus its changes indicate the glucuronizing capacity of the liver. The glucuronic acid pathway includes also enzymes not involved in drug metabolism, so en-

zyme induction cannot be calculated with certainty from the increase in D-glucaric elimination alone [15].

Owing to its pharmacokinetic and metabolic properties, antipyrine is suitable for assessing the capacity of the microsomal enzymes of the liver. Its elimination depends entirely on its metabolism in the liver; thus the half-life of plasma antipyrine reflects the function of the microsomal enzymes. As antipyrine does not bind to the plasma proteins but is evenly distributed in total body water, saliva is also suitable for measuring the concentration of the drug [21]. Thus, repeated hospitalization of the parents or the child was not necessary for the examination.

In our case the marked shortening of the antipyrine half-life, which was prolonged before flumecine treatment, the increased elimination of D-glucaric acid and the change in the bilirubin level have proving force in spite of the fact that we know of cases in which no significant difference in antipyrine half-life was found in G.d. patients as compared with controls [31].

Two months before the determination of antipyrine half-life gave phenobarbitural for enzyme induction with as a first approach, in the course of which the serum bilirubin level fell from 98 $\mu$mol/l to 25.6 $\mu$mol/l in 48 hours. This could also be considered diagnostic, although owing to fluctuations of the serum bilirubin level a chance coincidence may also have occurred.

The half-life of antipyrine in the mother, compared with the normal values given in the literature [21], showed moderately prolonged drug metabolism. In the father the half-life of antipyrine was at the upper limit of normal (Fig. 4). He complained of clinical symptoms from time to time, but so far this has not been substantiated by laboratory results.

The saccharose malabsorption in the mother deserves attention in view of the prolonged antipyrine half-life, even if we take into consideration that in adults this value is longer than in children. It seemed therefore that the mother is the carrier of both abnormal genes. It is generally accepted that G.d. is a genetically determined disease [1], with autosomal dominant inheritance. It would therefore be interesting to know the bilirubin UDP-glucuronyl transferase enzyme function in the younger child. This has, however, not been examined for lack of cooperation in gathering saliva. The incidence of inborn saccharose deficiency is not fully known (0.2–10%); it is inherited by autosomal recessivity [14].

To our knowledge antipyrine half-life and D-glucaric acid elimination have not been examined simultaneously previously, especially not before and after enzyme induction.

## REFERENCES

1. Bailey, A., Robinson, D., Dawson, A. M.: Does Gilbert's disease exist? Lancet **1**, 931–933 (1977)
2. Billing, B. H.: In: Familial Hyperbilirubinaemia. Okolicsanyi L. (ed.). Introduction to familial unconjugated hyperbilirubinaemia. John Wiley and Sons Ltd, Chichester 1980.

3. Billing, B. H., Black, M.: Bilirubin metabolism. Gut **10,** 250–254 (1979)
4. Black, M., Billing, B. H.: Hepatic bilirubin UDP-glucuronyl transferase activity in liver disease and Gilbert's syndrome. New Engl. J. Med. **280,** 1266–1271 (1971)
5. Bobori, J.: Hyperbilirubinaemia enzympathológiai alapon. Orv. Hetil. **102,** 407–409 (1961)
6. Brodie, B. B., Axelrod, I., Soberman, R., Levy, B. B.: The estimation of antipyrine in biological materials. J. Biol. Chem. **179,** 25–29 (1949)
7. Dawson, J., Carrl-Locke, D. L., Talbort, I. C., Rosenthal, F. D.: Gilbert's syndrome: evidence of morphological heterogeneity. Gut **20,** 848–853 (1979)
8. Felsher, B. F., Rickard, D., Redeker, A. G.: The reciprocal relation between caloric intake and the degree of hyperbilirubinemia in Gilbert's syndrome. New Engl. J. Med. **283,** 170–172 (1970)
9. McGee, J. O'D., Allan, J. G., Russel, R. I., Patrick, R. S.: Liver ultrastructure in Gilbert's syndrome. Gut **16,** 220–224 (1975)
10. Gógl, Á., Vezekényi, Zs., Simon, K., Jávor, T.: Enzyminductive effect of Zixoryn® in man. 5th Symposium on Clinical Pharmacology, Pécs, 1980. Abstract, p. 38.
11. Horváth, T., Gógl, Á., Ruzsa, Cs., Ludany, A., Jávor, T.: Drug-induced manifestation of hereditary hepatopathy. Acta Med. Acad. Sci. Hung. **31,** 219–228 (1974)
12. Jezequel, A. M.: The fine morphology of unconjugated hyperbilirubinaemia revisited with stereometry. In: Okolicsany, L. (ed.): Familial Hyperbilirubinaemia. John Wiley and Sons Chichester 1980, pp. 69–80.
13. Lapis, K.: The Gilbert-Meulengracht syndrome. In: Johannessen, J. V. (ed.): Electron Microscopy in Human Medicine. Vol. **8,** McGraw-Hill, New York 1979, pp. 70–73.
14. Lebenthal, E.: Textbook of Gastroenterology and Nutrition in Infancy. Raven Press, New York 1981, p. 937.
15. Lecamwasum, D. S.: Hepatic enzyme induction and its relationship to urinary D-glucaric acid excretion in man. Br. J. Clin. Pharmacol. **2,** 546 (1975)
16. Margittay, E.: Szűrővizsgálati adatok a Gilbert syndroma gyakoriságáról. Orv. Hetil. **112,** 266–268 (1971)
17. Marsh, C. A.: Metabolism of D-glucuronolactone in mammalian systems. Identification of D-glucaric acid as a normal constituent of urine. Biochem. J. **86,** 77–79 (1963)
18. Minio, F., Magnenat, P., Gautier, A.: L'ultrastructure du foie humain lors d'ictères non-hémolytiques chroniques congénitaux. Rev. Int. Hepatol. **16,** 323–327 (1966)
19. Németh, I., Szeleczki, T., Boda, D.: Koraszülöttek bilirubin szintjének emelkedése és D-cukorsav ürítése antenatalis dexamethason kezelés hatására. Gyermekgyógyászat **31,** 23–28 (1980)
20. Németh, I., Szeleczky, T., Boda, D.: Hyperbilirubinaemia and urinary D-glucaric acid excretion in premature infants following antepartum dexamethasone treatment. J. Perinat. Med. **9,** 35–39 (1981)
21. Németh, I., Szeleczki, T.: Enhanced drug metabolism and renal dysfunction. Br. J. Cin. Pharmacol. **11,** 92–95 (1981)
22. Okolicsanyi, L. (ed.): Familial Hyperbilirubinaemia. John Wiley and Sons, Chichester 1980.
23. Powell, L. W., Hemingway, E., Billing, B. H., Sherlock, S.: Idiopathic unconjugated hyperbilirubinaemia (Gilbert's syndrome). New Engl. J. Med. **277,** 1108–1112 (1967)
24. Schaff, Zs., Lapis, K., Sáfrányi, L.: Adatok a Gilbert-kór ultrastruktúrájához. Morph. Igazság. Orv. Szle. **10,** 17–26 (1970).
25. Schaff, Zs., Lapis, K., Sáfrányi, L.: Feinstrukturelle Veränderungen der Leber bei der Gilbert-Krankheit. Beitr. Path. Anat. **140,** 54–70 (1969)
26. Schmid, R.: Okolicsanyi, L. (ed.): Moderator's introduction to "familial desorders of hepatic bilirubin metabolism". John Wiley and Sons, Chichester 1980, pp. 87-89.
27. Simon, G., Varonier, H. S.: Étude au microscope électronique du foie de deux cas d'ictère non hémolitique congenital de type Gilbert. Schweiz. Med. Wochenschr. **93,** 459 (1963)
28. Simmons, C. J., Davis, M., Dordoni, B., Williams, R.: Urinary D-glucaric acid assay by an improved enzymatic procedure. Clin. Chim. Acta **51,** 47–50 (1974)
29. Tanikawa, K.: Liver pathology. In: Trump, F. B., Jones, T. T. (eds): Diagnostic Electron Microscopy. Vol. **2.** John Wiley and Sons, Chichester 1979, pp. 15–46.
30. Thompson, R. P. H.: Genetic transmission of Gilbert's syndrome. In: Okolicsanyi, L. (ed.): Familial Hyperbilirubinaemia. John Wiley and Sons, Chichester 1980, pp. 91–97.
31. Venuti, M., Nassuato, G., Lirussi, F.: Antipyrine and galactose kinetics in Gilbert's syndrome. In: Okolicsanyi, L. (ed.): Familial Hyperbilirubinaemia. John Wiley and Sons, Chichester 1980, pp. 181–186.

*Acta Medica Hungarica, 41 (4), pp. 247—252 (1984)*

# EFFECT OF PROSTACYCLIN ON THE MIXED FUNCTION MICROSOMAL OXIGENASE SYSTEM IN THE RAT LIVER

G. A. BÁLINT, Á. PAP, Gizella KARÁCSONY, V. VARRÓ, I. SZABÓ[1]

FIRST DEPARTMENT OF MEDICINE AND [1]INSTITUTE OF EXPERIMENTAL SURGERY, UNIVERSITY MEDICAL SCHOOL, SZEGED, HUNGARY

(Received May 24, 1983)

The microsomal fraction of the liver was studied for protein and cytochrome $P_{450}$ contents as well as for aminopyrine-N-demethylase and aniline-hydroxylase activity and for its BIC spectrum under the effect of PG–$I_2$ treatment. A significant increase was found in the cytochrome $P_{450}$ content after short term treatment, while continuation of PG–$I_2$ administration caused a significant and long lasting decrease of cytochrome $P_{450}$. After prolonged application of PG–$I_2$ the aniline-hydroxylase content decreased significantly, while the aminopyrine-N-demethylase enzyme showed no change. The BIC spectrum after 80 days PG–$I_2$ treatment significantly decreased in the 431–432 nm region, corresponding to the decrease of cytochrome $P_{450}$. Long term PG–$I_2$ application caused no detectable ultrastructural changes in the liver.

**Keywords:** mixed function oxigenases, prostacyclin, rat, liver

**Abbreviation:** MFO: mixed function oxigenases; PG–$I_2$: prostacyclin; BIC: butylisocyanide; SER: smooth endoplasmic reticulum; GER: granulated endoplasmic reticulum

## Introduction

The intensity of toxic manifestations of a drug depends first of all on the dosage and the duration of treatment. Activities of the hepatic detoxicating enzymes or MFO determine the nature and fate of drugs in the animal organism [7].

Exposure of mammals to drugs may result in an increase in the size of the liver. The increase is accompanied by an increase in the synthesis of proteins; this occurs mostly, but not entirely in the SER of the hepatocytes. These alterations in hepatic ultrastructure and biochemistry increase the concentration of MFO in microsomes in the SER.

The threshold dosage for induction of MFO depends on the chemical nature of the inducer and on the mode of administration. Although a large

Send offprint requests to G. A. Balint, P.O. Box 469, H-6701 Szeged, Hungary

number of compounds of different molecular types may increase the activity of the same enzyme-system, there are a number of enzymes which are increased by a certain class of compounds but not by others. Ultrastructurally, the increase in SER-membranes is reflected in an increase in number and size of microsomal components such as proteins, enzymes, etc. [15].

A moderate enlargement of the liver, i.e. of the individual hepatocytes, is beneficial if it is the result of an increase of SER and MFO [16].

The excess of SER, forming tightly packed clusters of tubular membranes with no glycogen and little hyaloplasm together with damaged mitochondrial membranes, may be considered a criterion of toxic injury [4].

The hypertrophy and growth of SER may involve different processes. The gross protein synthesis which goes in parallel with the increase in MFO activity in the early stages of induction probably comprises changes in the rates of synthesis of many proteins [6].

Since microsomal enzymes form a small part of total cell proteins, even marked changes in their rates of synthesis cannot account for the gross changes in protein synthesis and vice versa. Moreover some of the inducers, for example phenobarbital, have a small but significant effect at the ribosomal and nuclear level too [10].

Considering that PG–$I_2$ is one of the possible future drugs, and we have presented data suggesting its interaction with protein-synthesis [1, 2], in the present work the effect of PG–$I_2$ treatment on the MFO system as a model experiment, has been studied.

## Materials and methods

Female Wistar rats weighing 220–270 g were used. Either on the same day or 24 hours after the last treatment with PG–$I_2$ the animals were sacrificed, their liver was removed and processed for the preparation of microsomal fraction by Pap's method as described earlier [12, 13, 14]. Cytochrome $P_{450}$ was measured according to Omura and Sato [11], microsomal protein according to Lowry et al. [8]. Aniline-hydroxylase activity was estimated by measurement of the formed p-aminophenol, according to Imai et al. [5], aminopyrine-N-demethylase activity by that of formaldehyde, according to Nash [9]. Investigation of BIC spectrum was performed according to Pap and Szarvas [14].

Immediately after removal of the liver, specimens from the right lobe were taken for electron-microscopic investigation.

The removed liver specimens of about 1 $mm^3$ in volume were fixed at 0–4 °C in 4% glutaraldehyde and 1% osmiumtetroxide solution with 0.15 M phosphate-buffer as solvent. They were dehydrated in an ascending alcohol series, and stained with uranyl-acetate dissolved in 70% ethanol, embedded in Araldite and stained with lead citrate. Sections were cut with a Reichert-ultramicrotome and studied by a Tesla 500 BS electron microscope.

A stock solution of PG–$I_2$ (1 mg/ml) was made up freshly in 0.05 M Tris-buffer, pH 9.6. Dilutions were made immediately prior to use in ice-cold isotonic sodium-bicarbonate solution, and given either orally by gastric tube or intraperitoneally, in a single dose of 100 $\mu$g/kg. The control animals received normal saline in appropriate amounts.

Within each animal group mean $\pm$ S.E.M. was calculated. Statistical analysis vs. the control group was performed with Student's *t*-test. Significant differences were assumed when the probability was less than 5%.

## Results

Experimental results are summarized in Tables I, II and III.

According to the present results PG–$I_2$ treatment had a double effect on MFO.

Shortly after PG–$I_2$ administration the amount of cytochrome $P_{450}$ was significantly higher than in the control animals. Continuing the daily PG–$I_2$ administration the amount of cytochrome $P_{450}$ decreased, first to the

**Table I**

*Effect of prostacyclin on the mixed function microsomal oxigenase system in the rat liver*
*Group I. Short term treatment*

| Treatment 3 × 100 μg/kg i.p. daily | n | Body weight g | Liver weight g | Microsomal protein, mg/g wet liver | Cytochrome $P_{450}$ nmol/mg microsomal prot. | Cytochrome $P_{450}$ nmol/g wet liver |
|---|---|---|---|---|---|---|
| Control | 28 | 214±5.1 | 7.27±1.26 | 22.65±1.64 | 0.763±0.143 | 16.829±2.093 |
| 1 + (0)! | 8 | 235±6.3 | 6.72±0.98 | 24.95±1.05 | 0.680±0.162 | 16.923±4.100 |
| 1 + (1) | 8 | 225±7.8 | 6.37±1.17 | 22.43±1.49 | 1.232±0.490** | 27.418±2.169** |
| 3 + (0) | 8 | 228±8.9 | 8.91±1.83 | 22.60±2.03 | 0.828±0.229 | 18.681±3.564 |
| 3 + (1) | 8 | 268±7.0 | 9.52±0.66 | 22.75±0.84 | 0.637±0.035 | 14.484±1.798 |
| 7 + (0) | 8 | 237±5.9 | 8.90±1.14 | 16.71±0.60 | 0.858±0.222 | 14.242±4.160 |
| 7 + (1) | 8 | 245±6.9 | 7.95±1.49 | 22.67±1.32 | 0.401±0.040** | 8.923±1.669** |
| 9 + (0) | 8 | 207±4.9 | 8.64±1.08 | 28.28±1.39 | 0.270±0.039** | 7.426±0.819** |

mean ± S.E.M.
** $P < 0.01$ vs. control
n = number of rats
! = first number: duration of treatment in days;
numbers in parentheses: (0) sacrifice on same day,
(1) sacrifice 24 hours after last treatment.

**Table II**

*Effect of prostacyclin on the mixed function microsomal oxigenase system in the rat liver*
*Group II. Long term treatment*

| Treatment 100 μg/kg p.o. daily | n | Body weight g | Liver weight g | Microsomal protein mg/g wet liver | Cytochrome $P_{450}$ nmol/mg microsomal prot. | Cytochrome $P_{450}$ nmol/g wet liver |
|---|---|---|---|---|---|---|
| Control | 15 | 264±8.6 | 9.32±0.79 | 24.18±0.62 | 0.522±0.018 | 12.528±0.279 |
| 80 + (1)! | 15 | 261±7.9 | 9.18±0.92 | 24.70±0.35 | 0.453±0.015* | 11.154±0.343* |

mean ± S.E.M.
* $P < 0.05$ vs. control
n = number of rats
! = first number: duration of treatment in days;
number in parentheses: (1) sacrifice 24 hours after last treatment

normal level, and after one week treatment significantly below it. This finding is in complete agreement with our previous result, in that first PG–$I_2$ shortens the barbiturate sleeping time, then after a few days treatment this effect disappears [3]. The subnormal level of cytochrome $P_{450}$ seemed to persist as even after 80 days treatment it was still significantly below the normal level.

It seems that the first phase of the effect is due to a quick response of the liver, either with a rapid enhancement of synthesis or a depletion of the stored enzyme. Later a plateau below the normal value is reached, indicating a new steady state. It is not clear why the new level is lower than the normal one, whether it is due to an enhanced breakdown or a diminished rate of synthesis.

**Table III**

*Effect of prostacyclin on the mixed function microsomal oxigenase system in the rat liver*

| Treatment 100 μg/kg p.o. daily | n | Body weight g | Liver weight g | Aminopyrine-N-demethylase, nmol/g liver/30 min formaldehyde | Aniline-hydroxylase, nmol/g liver/30 min p-aminophenol |
|---|---|---|---|---|---|
| Control | 8 | 252±6.8 | 8.64±1.13 | 1352.86±75.80 | 287.86±13.49 |
| 80 + (1)[!] | 8 | 248±6.1 | 8.39±1.19 | 1313.75±67.80 | 256.88± 8.18* |

mean ± S.E.M.
* $P < 0.05$ vs. control
n = number of rats
[!] = first number: duration of treatment in days;
number in parentheses: (1) sacrifice 24 hours after last treatment

During PG–$I_2$ treatment, whether short or prolonged, aminopyrine-N-demethylase showed no change, while on its prolonged application aniline-hydroxylase decreased significantly.

The BIC spectrum in the 431–432 nm region decreased significantly after 80 days. This type of change corresponds to the decrease of cytochrome $P_{450}$, while the unchanged spectrum at the 455–456 nm region indicates that PG–$I_2$ had no effect on cytochrome $P_{448}$ (Figs 1 and 2).

The ultrastructural alterations after a short treatment also correspond to the phenomenon observed with hexobarbital sleeping time, i.e. first a normal ultrastructure could be observed with a moderate hypertrophy of the SER, then later a disappearance of glycogen granules, with normal cell structure, characterized the electron microscopic picture [3]. Prolonged (80 days) treatment caused no ultrastructural changes in the liver (Fig. 3).

The present results call attention to some hitherto unknown steps in the mechanism of action of PG–$I_2$, suggesting at the same time that PG–$I_2$ has no hepatotoxic properties.

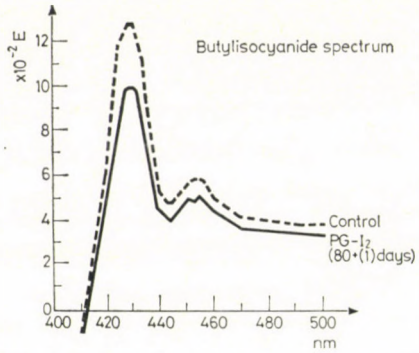



*Fig. 1.* Modification of BIC spectrum induced by long term PG–$I_2$ treatment (the graph represents mean values for two animals)

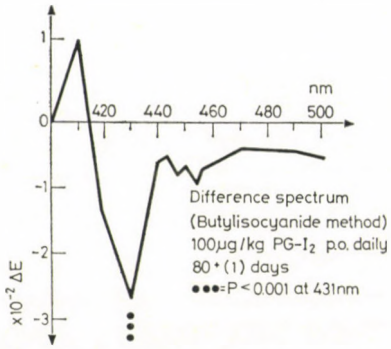



*Fig. 2.* BIC difference spectrum of the microsomal fraction of rat liver. The graph represents mean ± S.E.M. for two animal groups (n=15/group). The mean value of the (untreated) control group has been reduced to zero (Bar = S.E.M.). The difference between the mean values of the (reduced) control and PG–$I_2$ treated groups is given as the difference spectrum (Bar = S.E.M.)

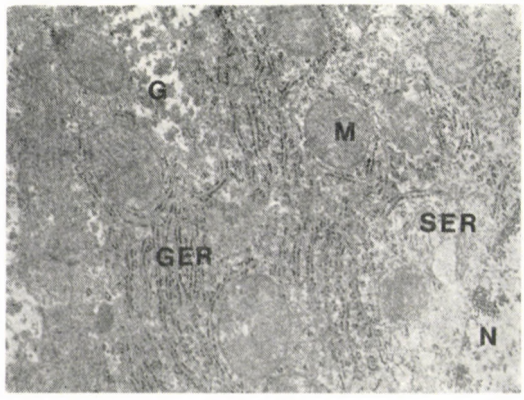



*Fig. 3.* Ultrastructural picture of rat liver after 80 + (1) days treatment with PG–$I_2$. ×10 000

## Acknowledgements

The authors are indebted to Mrs K. Korom and Miss E. Barna for skilful technical assistance. * This work was supported by Ministry of Health Research Grant No: TPB–EüM–46.

## REFERENCES

1. Balint, G. A., Varró, V.: Actinomycin inhibits gastric mucosal protection by prostacyclin in rats. Prostaglandins **21,** 255–257 (1981)
2. Balint, G. A., Varró, V.: Prostacyclin and protein synthesis inhibitors. Interaction on experimental gastric ulcer. 7th World Congress of Gastroenterology, Stockholm 1982. In: Scand. J. Gastroenterol. **17,** (Suppl. 78.) 42 (1982)
3. Balint, G. A., Varró, V.: Prostacyclin and protein synthesis. Acta Physiol. Acad. Sci. hung. **61,** 115–122 (1983)
4. Hutterer, F., Schaffner, F., Kloin, F. M., Popper, H.: Hypertrophic, hypoactive endoplasmic reticulum; a sensitive indicator of hepatotoxicity exemplified by dieldrin. Science, **161,** 1077–1079 (1968)
5. Imai, A., Ito, A., Sato, R.: Evidence for biochemically different types of vesicles in the hepatic microsomal fraction. J. Biochem. **60,** 417–428 (1966)
6. Kato, R., Chiesara, E., Vassanelli, P.: Further studies on the inhibition and stimulation of microsomal drug-metabolizing enzymes of rat liver by various compounds. Biochem. Pharmacol. **13,** 69–83 (1964)
7. Khan, M. A. Q.: Induction of drug-metabolizing enzymes by insecticides and other xenobiotics. Pharmac. Ther. **11,** 43–107 (1980)
8. Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J.: Protein measurement with the Folin phenol reagent. J. biol. Chem. **193,** 265–275 (1951)
9. Nash, T.: The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. Biochem. J. **55,** 416–421 (1953)
10. Omura, T., Kuriyama, Y., Siekevitz, P., Palade, G. E.: Effect of phenobarbital on the turnover of microsomal enzymes. In: Gillette, J. R., Conney, A. H., Cosmides, G. H., Estabrook, R. W., Fouts, J. R., Mannering, G. J. (eds): Microsomes and Drug Oxidations. Academic Press, New York 1969, pp. 475–494.
11. Omura, T., Sato, R.: The carbon monoxide binding pigment of liver. J. biol. Chem. **239,** 2370–2378 (1964)
12. Pap, Á.: Analyse de l'induction des enzymes microsomiales par le 3-trifluométhyle-alpha-éthyle-benzhydrol (RGH-3332). Thérapie, **34,** 635–640 (1979)
13. Pap, Á., Szarvas, F.: Májkárosodás, fenobarbital, nor-androstenolon-fenilpropionát, illetve chloramphenicol kezelés hatása a szerves foszforsavészter mérgezések lefolyására. Kísérl. Orvostud. **28,** 172–179 (1976)
14. Pap, Á., Szarvas, F.: Effect of alpha-pinene on the mixed function microsomal oxidase system in the rat. Acta med. Acad. Sci. hung. **33,** 379–385 (1976)
15. Ragnotti, G., Aletti, M. G.: Phenobarbitone-induced stimulation of protein synthesis in liver of diabetic rats. Biochim. biophys. Acta (Anest.) **521,** 352–362 (1978)
16. Schulte-Hermann, R.: Induction of liver growth by xenobiotic compounds and other stimuli. Crit. Rev. Toxicol. **3,** 97–158 (1974)

*Acta Medica Hungarica, 41 (4), pp. 253–258 (1984)*

## *Gastroenterology*

# THE GASTRIC FUNDIC MUCOSAL ENERGY SYSTEMS AND THE HCl-INDUCED LESIONS IN RATS IN DEPENDENCE ON STARVATION

F. Morón, L. Nagy, Gy. Mózsik

FIRST DEPARTMENT OF MEDICINE, UNIVERSITY MEDICAL SCHOOL, PÉCS, H-7643, HUNGARY

(Received July 18, 1983)

Gastric mucosal lesions were produced by intragastric administration of 0.6 M HCl (1 ml) to rats starved for 48 or 24 h, and to unstarved rats which received 5 or 20% glucose solution ad libitum. The number and severity of gastric lesions were recorded and the mucosal levels of ATP, ADP, and AMP were enzymatically measured, cAMP was determined by RIA. Adenylate pool, energy charge, and ATP · $ADP^{-1}$ were rated.

It has been found that: the 20% glucose fed rats showed the highest levels of biochemical constituents, but the lowest number and severity of gastric lesions; there were significant negative correlations between the number and severity of lesions and the mucosal levels of ADP, AMP and cAMP; significant positive correlations were found between the number and severity of gastric lesions and the ratios of ATP · $ADP^{-1}$ and energy charge. It has been concluded that: the energy turnover processes of the fundic mucosa were significantly higher in the animals fed 20% glucose; the increased energy turnover in the gastric mucosa may produce a better metabolic adaptation against the necrotizing effect of HCl.

**Keywords:** acute starvation, HCl, gastric mucosal lesions, energy systems, ATP, ADP, AMP, cAMP, ATP · $ADP^{-1}$, energy charge

## Introduction

The essential role of the energy systems of the gastric fundic mucosa has been shown in the development of gastric mucosal lesions (ulcers) [6, 7, 8, 9, 10, 11, 12] and in gastric cytoprotection [13, 14].

The relationship between the extent of gastric lesions and acute starvation has been demonstrated in rabbits in haemorrhagic shock [6] and with gastric mucosal lesions in rats induced by aspirin, epinephrine or pyloric ligation [15].

Previously we have shown that the fundic mucosal levels of ATP, AMP, cAMP, and of the ratio of ATP · $ADP^{-1}$ decreased significantly, while the

Send offprint requests to: Gy. Mózsik. First Department of Medicine, University Medical School, H-7643 Pécs, Hungary.

levels of ADP and lactate increased, during development of gastric mucosal lesions (ulcers) induced by intragastric administration of 0.6 M HCl (1 ml) in rats [8, 13, 14].

The aims of the present paper were:

— to study the relationship between the membrane-bound energy systems of the gastric fundic mucosa and the development of lesions produced by intragastric administration of 0.6 M HCl in starved and unstarved rats;

— to evaluate whether correlations could be noted between the membrane-bound energy ATP-dependent systems and the extent of the gastric mucosal lesions (ulcers) induced by intragastric administration of 0.6 M HCl in rats.

## Materials and methods

The experiments were carried out in CFY strain rats of both sexes, weighing 180 to 210 g. Female and male rats were homogeneously allocated by random selection into different groups.

The animals were starved for 48 hours (Group I) or 24 h (Group II). These rats received water ad libitum. The others had free access to 5% (Group III) or 20% (Group IV) glucose solution for 24 h.

The gastric mucosal lesions were produced by intragastric administration of 0.6 M HCl (1 ml) according to Robert et al. [16]. The rats were killed by an ether overdose 1 hour later. The experiments were done between 8 and 10 a.m. The stomachs were quickly dissected out and opened along the lesser curvature. Samples from the gastric fundic mucosa were scarped off and frozen in liquid nitrogen.

The number of gastric lesions was noted and their severity scored as: *grade 0*, no lesion; *grade 1*, lesion smaller than 1 mm; *grade 2*, between 1 and 2 mm; *grade 3*, 2 to 3 mm; *grade 4*, 3 to 4 mm; and *grade 5*, greater than 4 mm. Number and sum of gastric lesions scores were calculated for each animal [13].

The tissue levels of adenosine triphosphate (ATP), [3], adenosine diphosphate (ADP) [4], adenosine monophosphate (AMP) [4] and lactate [2] were measured enzymatically (Boehringer Biochemical Test, Mannheim, FRG). The tissue levels of cyclic adenosine monophosphate (cAMP) were measured by radioimmunoassay (RIA) (Becton Dickinson Co., USA). The extent of phosphorylation and/or dephosphorylation was evaluated according to Atkinson's formula [1]: $(ATP + 0.5\ ADP) \cdot (ATP + ADP + AMP)^{-1}$. The biochemical compounds were calculated in relation 1.0 mg of gastric mucosal protein. Protein was measured by biuret reaction [5].

Statistical analysis: analysis of variance was performed previously. The comparison of groups was done by Student's unpaired "t" test, Mann–Whitney's test was employed to process data for gastric lesion severity. The study of correlations was done by the method of two variable lineal regression.

The results are expressed as means $\pm$ S.E.M.

## Results

Table I summarizes all data and Table II shows the levels of significance in relation to the different groups. It has been found that: (1) the number and severity of gastric mucosal lesions were significantly higher in the rats starved for 48 or 24 hours and in those fed 5% glucose than in those receiving 20% glucose; (2) the tissue levels of ATP, ADP, AMP, adenylate pool, and cAMP were significantly decreased in 48 and 24 hours starved rats in contrast with

the unstarved animals; (3) the energy charges in 48 and 24 hours starved rats were significantly higher than those in the unstraved rats; (4) the animals fed 20% glucose showed significantly highest levels of the different biochemical compounds, while the number and severity of gastric lesions were the lowest.

**Table I**

*Development of gastric mucosal lesions produced topical (intragastric) HCl administration and the energy systems of the gastric fundic mucosa in rats*

| Variables | Starved rats | | Fed rats | |
|---|---|---|---|---|
| | 48 hrs (n=10) | 24 hrs (n=10) | 5% glucose (n=10) | 20% glucose (n=10) |
| Number of lesions | 11.6±1.5* | 10.2±1.3 | 8.6±1.0 | 3.9±1.1 |
| Severity of lesions | 44.8±6.1 | 36.6±6.9 | 29.6±5.1 | 13.4±4.3 |
| ATP[1] | 17.0±2.5 | 16.1±2.0 | 22.1±1.6 | 40.0±6.1 |
| ADP[1] | 1.2±0.1 | 1.4±0.1 | 5.6±0.9 | 6.1±1.2 |
| ATP.ADP$^{-1}$ | 14.2±1.4 | 11.5±1.0 | 4.9±0.7 | 8.1±1.2 |
| AMP[1] | 0.4±0.1 | 0.5±0.16 | 3.2±0.8 | 5.1±0.6 |
| ATP + ADP + AMP[1] | 18.6±2.4 | 18.0±2.0 | 30.9±2.4 | 51.2±7.2 |
| Energy charge | 0.95±0.02 | 0.94±0.01 | 0.81±0.02 | 0.82±0.02 |
| cAMP[2] | 4.5 ±0.4 | 6.2 ±0.2 | 7.8 ±0.8 | 10.6 ±1.2 |

* Values are means ± S.E.M.; "n" means the number of animals
[1] Results are expressed in nanomoles · mg protein$^{-1}$
[2] Results are expressed in picomoles · mg protein$^{-1}$

**Table II**

*Statistical significances of the variables in Table I*

| Variables | I–II | I–III | I–IV | II–III | II–IV | III–IV |
|---|---|---|---|---|---|---|
| Number of lesions | NS* | NS | <0.001 | NS | <0.01 | <0.01 |
| Severity of lesions | NS | NS | <0.001 | NS | <0.05 | <0.05 |
| ATP | NS | NS | <0.01 | <0.05 | <0.01 | <0.05 |
| ADP | NS | <0.001 | <0.001 | <0.001 | <0.001 | NS |
| ATP · ADP$^{-1}$ | NS | <0.001 | <0.05 | <0.001 | <0.05 | <0.05 |
| AMP | NS | <0.01 | <0.001 | <0.01 | <0.001 | NS |
| ATP + ADP + AMP | NS | <0.001 | <0.001 | <0.001 | <0.001 | NS |
| Energy charge | NS | <0.001 | <0.001 | <0.001 | <0.001 | NS |
| cAMP | <0.01 | <0.01 | <0.001 | NS | <0.01 | NS |

I = starved for 48 h; II = starved for 24 h; III = fed 5% glucose; IV = fed 20% glucose
* non significant

As to the correlations, significant negative correlations were found between the extent of gastric mucosal lesions (number and severity) and the fundic mucosal levels of ATP, AMP, and cAMP; and there were significant positive correlations between the extent of gastric mucosal lesions and the ratios of ATP · ADP$^{-1}$ and energy charge (Table III).

**Table III**

*Correlations of the biochemical parameters measured in the gastric fundic mucosa and number and severity of gastric mucosal lesions (ulcers) induced by intragastric administration of 0.6 M HCl*

| X (mean ± S.E.M.) / Y₁ (mean ± S.E.M.) | Regression line | $r_1$ | $Y_2$ (mean ± S.E.M.) (30.6±3.2) Regression line | $r_2$ |
|---|---|---|---|---|
| ATP[1] (24.1±2.4) | $Y_1 = 9.93 - 0.06\times$ | −0.20 | $Y_2 = 35.95 - 0.22\times$ | −0.17 |
| ADP[1] (3.4±0.5) | $Y_1 = 9.91 - 0.41\times$ | −0.32* | $Y_2 = 36.51 - 1.67\times$ | −0.31* |
| ATP · ADP$^{-1}$ (10.3±1.0) | $Y_1 = 5.87 + 0.25\times$ | +0.36* | $Y_2 = 18.92 + 1.13\times$ | +0.38* |
| AMP[1] (2.2±0.4) | $Y_1 = 10.25 - 0.78\times$ | −0.45** | $Y_2 = 38.66 - 3.48\times$ | −0.47** |
| ATP+ADP+AMP[1] (29.8±3.0) | $Y_1 = 10.54 - 0.07$ | −0.28 | $Y_2 = 38.73 - 0.27$ | −0.25 |
| Energy charge (0.88±0.01) | $Y_1 = 10.88 + 21.93\times$ | +0.41** | $Y_2 = 50.96 + 92.52\times$ | +0.41** |
| cAMP[2] (7.3±0.5) | $Y_1 = 11.51 - 0.41$ | −0.29 | $Y_2 = 44.81 - 1.91\times$ | −0.32* |

$Y_1$ = number of gastric lesions; $Y_2$ = severity of gastric lesions; $r_1$ and $r_2$ = coefficients of regression; + = $P < 0.05$ and ++ = $P < 0.01$.
[1] results are expressed nmol · mg protein$^{-1}$; [2] results are in picomoles · mg protein$^{-1}$; Number of animals (n=41) · r = 0.30 when P = 0.05.

The tissue lactate level was 0.736±0.158 nmol/mg protein$^{-1}$ in 48 hours starved rats, and 0.582±0.090 nmol/mg protein$^{-1}$ in the 20% glucose fed animals. There was not significant difference in lactate level between these groups ($P > 0.05$).

## Discussion

The energy systems in the fundic gastric mucosa have been shown to be closely related to the development of gastric mucosal lesions [6–12]. Our previous observations have suggested that ulcerogenesis and gastric cytoprotection are very dynamic metabolic processes [8–14].

The present results suggest an important role of the membrane-bound ATP-dependent energy systems in the development of gastric mucosal lesions induced by 0.6 M HCl in starved and unstarved rats.

The fundic ATP level is regulated by the balance between ATP resynthesis and ATP breakdown into ADP (by membrane ATPase) and/or into cAMP (by adenylate cyclase). ATP resynthesis may decrease under hypoxaemic circumstances.

In the starved animals, ATP breakdown into ADP and cAMP was decreased. The lowest ATP breakdown into ADP and cAMP was supported

by: (1) the low levels of ADP and cAMP; (2) the high ratio of ATP · $ADP^{-1}$ and (3) the high ratio of energy charge in the starved rats. These facts indicated that the adenosine compounds were practically phosphorylated, in this form of ATP.

ATP resynthesis seemed to be diminished in the starvated rats as ATP level was low in the fundic gastric mucosa. In spite of this we could not conclude to there being a hypoxaemic situation because there was no significant difference in the lactate level here and in the rats fed 20% glucose.

The number and severity of gastric mucosal lesions were higher in the starved rats than in those fed 20% glucose. The present data suggest that starved rats have a low energy turnover as a protecting mechanism against starvation, but this metabolic state does not seem to be a suitable one to defend the gastric fundic mucosa from the necrotizing effect of HCl.

In the rats fed 20% glucose, the fundic levels of ATP, ADP, AMP, and cAMP were high indicating a higher extent of ATP breakdown into both ADP and cAMP. Moreover, the adenylate pool increased significantly and the energy charge diminished, pointing a higher energy liberation than in the starved animals. If the breakdown increases and the level of ATP is high, the extent of oxidative phosphorylation could also increase. The increased ATP resynthesis excludes the presence of hypoxaemic damage in the gastric fundic mucosa.

All these results suggest that starved rats can liberate less energy in their gastric fundic mucosa than do animals fed 20% glucose.

Furthermore, significant negative correlations have been found between the number and severity of gastric mucosal lesions (ulcers) and the mucosal level of ADP, AMP, and cAMP; and significant positive correlations between the number and severity of gastric lesions and the ratio of ATP · $ADP^{-1}$ and energy charge. The lack of significance in this case of ATP (negative) and adenylate pool (positive) may suggest that the extent of lesions was not related merely to the amount of ATP and adenylate pool in the tissue, but it correlated also with ATP breakdown and resynthesis.

Our results thus suggest that the increased energy turnover in the rat gastric fundic mucosa may improve the metabolic adaptation against the necrotizing effect of HCl. This fact may have clinical importance as the ulcer eliciting factors are dynamic and the metabolic ability of the gastric fundic mucosa might act as a protecting factor.

## REFERENCES

1. Atkinson D. E.: The energy charge of the adenylate pool as regulatory parameter. Interaction with feedback modifiers. Biochemistry 7, 4030–4034 (1968)
2. Gutmann, I., Wahlefeld, A. W.: L—(+)-lactate determination with lactate dehydrogenase and NAD. In: Bergmeyer, H. U. (ed.). Methods of Enzymatic Analysis. Vol. 3. 2nd Ed., Academic Press, Inc., New York 1974, pp. 1464–1468.

3. Jaworekk, D., Gruber, W., Bergmeyer, H. U.: Adenosine-5'-triphosphate. Determination with 3-phosphoglycerate kinase. In: Bergmeyer, H. U. (ed.). Methods of Enzymatic Analysis. Vol. 4. 2nd Ed. Academic Press Inc., New York and London, 1974, pp. 2097–2101.
4. Jaworek, D., Gruber, W., Bergmeyer, H. U.: Adenosine-5'-diphosphate and adenosine-5'-monophosphate. In: Bergmeyer, H. U. (ed.). Methods of Enzymatic Analysis. Vol. 4. 2nd Ed., Academic Press Inc., New York–London 1974, pp. 2127–2131.
5. Layne, E.: Spectophotometric and turbidimetric methods for measuring proteins. In: Colowich, S. P., Kaplan, O. (eds). Methods in Enzymology. Vol. 3. Academic Press Inc., New York–London, 1957, pp. 447–454.
6. Menguy, R., Master, Y. F.: Mechanism of stress ulcer. IV. Influence of fasting on the tolerance of gastric mucosal energy metabolism to ischemic and on the incidence of stress ulceration. Gastroenterology **66,** 1177–1186 (1974)
7. Menguy, R.: Role of the gastric mucosal energy metabolism in the etiology of stress ulceration. World J. Surgery **5,** 175–180 (1981)
8. Morón, F., Mózsik, Gy., Jávor, T., Fiegler, M., Nagy L., Patty, I., Tárnok, F.: Membrane-bound ATP-dependent energy systems and the development of the gastric mucosal damage in rats produced by 0.2 M NaOH, 25% NaCl, 0.6 M HCl and 96% ethanol. Int. J. Tiss. React. **4,** 357—362 (1983)
9. Mózsik, Gy., Kiss, B., Krausz, M., Jávor, T.: A biochemical-cellular-morphological explanation of the development of gastric ulcer in Shay rats. Scand. J. Gastroent. **4,** 633–640 (1969)
10. Mózsik, Gy., Jávor, T., Hauck, M., Karsai, T.: On some cellular mechanism of ulcer development in pylorus-ligated rats. In: Pfeiffer, C. J. (ed.). Peptic Ulcer. Lippincott Co., Philadelphia, 1971, pp. 171–184.
11. Mózsik, Gy., Nagy, L., Tárnok, F., Vizi, F.: The energy systems of gastric tissues, their neural, hormonal and pharmacological regulations in order to gastric $H^+$ secretion and ulcerogenesis. A review of animal experiments and clinical biochemical studies. Acta medica Acad. Sci. Hung. **36,** 1–29 (1979)
12. Mózsik, Gy., Fiegler, M., Nagy, L., Patty, I., Tárnok, F.: Gastric and small intestinal energy metabolism in mucosal damage. In: Mózsik, Gy., Hänninen, O., Jávor, T. (eds). Advances in Physiological Sciences. Vol. 29. Gastrointestinal Defence Mechanisms. Pergamon Press, Oxford — Akadémiai Kiadó, Budapest, 1981, pp. 213–276.
13. Mózsik, Gy., Morón, F., Jávor, T.: Cellular mechanisms of the development of gastric mucosal damage and of gastrocytoprotection induced by prostacyclin in the rats. A pharmacological study. Prostaglandins, Leucotrienes, Medicine **9,** 71–84 (1982)
14. Mózsik, Gy., Morón, F., Fiegler, M., Jávor, T., Nagy, L., Patty, I., Tárnok, F.: Interrelationships between membrane-bound ATP-dependent energy systems, gastric mucosal damage produced by NaOH, hypertonic NaCl, HCl and alcohol, and prostacyclin-induced gastric cytoprotection in rats. Prostaglandins, Leucotrienes, Medicine 12, 423—426 (1983)
15. Nagy, L., Fiegler, M., Jávor, T., Mózsik, Gy.: Role of acute starvation on the development of different experimental ulcers in the rats. In: Mózsik, Gy., Hänninen, O., Jávor, T. (eds). Advances in Physiological Sciences. Vol. 29. Gastrointestinal Defence Mechanisms. Pergamon Press, Oxford — Akadémiai Kiadó, Budapest, 1981, pp. 117–126.
16. Róbert, A., Nemazis, J. E., Lancaster, C., Hanchar, A. J.: Cytoprotection by prostaglandins in rats. Prevention of gastric necrosis produced by alcohol, HCl, NaOH, hypertonic NaCl and thermal injury. Gastroenterology **77,** 433–443 (1979)

*Acta Medica Hungarica, 41 (4), pp. 259–261 (1984)*

# PROSTACYCLIN AND DNA-SYNTHESIS INHIBITORS: THEIR INTERACTION ON EXPERIMENTAL GASTRIC ULCER IN THE RAT

G. A. BÁLINT and V. VARRÓ

FIRST DEPARTMENT OF MEDICINE, UNIVERSITY MEDICAL SCHOOL, 6701 SZEGED, KORÁNYI SÉTÁNY 8, HUNGARY

(Received July 14, 1983)

The protective effect of prostacyclin on gastric mucosal damage by indomethacin or restraint in rats was partly inhibited by DNA-synthesis inhibitors. Protectin by prostacyclin may therefore depend upon intact DNA-synthesis.

**Keywords:** prostacyclin, DNA-synthesis inhibitors, experimental gastric ulcer

**Abbreviations:** $PG\text{–}I_2$: prostacyclin, DTIC: dacarbazine
CYAR: cytosine arabinoside,
RUMY: rubidomycin

## Introduction

It was reported previously [1] that actinomycin inhibits gastric mucosal protection by $PG\text{–}I_2$ in rats. In the present study we examined the interaction between $PG\text{–}I_2$ and different DNA-synthesis inhibitors on indomethacin and stress induced gastric ulceration in rats.

## Materials and methods

Twenty-four groups of female Wistar rats weighing 210–270 g were fasted for 24 hours but allowed water ad libitum.

The models used and the mode of evaluation was published elsewhere [1].

Within each group (n=10/group) mean values ±S.E.M. were calculated and analysed statistically using Student's *t*-test. Significant differences were assumed when the probability was less than 5%.

The following DNA-synthesis inhibitors were tested: DTIC (Dome Laboratories, Stoke Poges, England), 2 mg/kg, i.p.; CYAR (Cytosar®, Upjohn, Kalamazoo, USA), 50 mg/kg, i.p.; RUMY (Rubomycin® Medexport, Moscow, SU) 1 mg/kg, i.p.

$PG\text{–}I_2$ was dissolved (1 mg/ml) freshly in 0.05 m Tris-buffer pH 9.6. Dilutions were made immediately prior to use in ice-cold isotonic sodium bicarbonate solution and injected i.p. in 100 μg/kg single doses.

Results are expressed as mean ± S.E.M.

Send offprint requests to: G. A. Bálint P.O. Box 469. H-6701 Szeged, Hungary

## Results and discussion

Results are summarized in Table I. It has been reported earlier that actinomycin (I) and other protein synthesis inhibitors [2, 3] such as cycloheximide, puromycin and kanamycin — inhibit the cytoprotective effect of PG–$I_2$.

The inhibition by different substances of the cytoprotective effect of PG–$I_2$ in different experimental ulcers supported the conclusion that the protective effect requires new proteins. Considering that the first steps of protein synthesis are connected with DNA, investigation of the effect of different DNA-synthesis inhibitors promised to be an intriguing problem.

**Table I**

*Prostacyclin and DNA-synthesis inhibitors: interaction on experimental gastric ulceration*

| Group | Ulcer Index (mean ± S.E.M.) | | | |
|---|---|---|---|---|
| | Control | DNA Inhibitor | PG–$I_2$ | PG–$I_2$ + DNA Inhibitor |
| DTIC | | | | |
| Indomethacin | 10.7±5.1 | 18.1±4.7 | 5.2±2.2 | 7.7±4.3 |
| Stress | 13.7±5.8 | 11.6±5.7 | 5.7±2.7 | 3.4±1.5 |
| CYAR | | | | |
| Indomethacin | 21.1±3.4 | 17.9±3.3 | 4.2±1.4*** | 16.2±4.9" |
| Stress | 12.1±3.9 | 12.3±4.9 | 3.6±0.5* | 8.8±2.4" |
| RUMY | | | | |
| Indomethacin | 25.1±4.1 | 18.0±5.1 | 9.3±2.7* | 3.7±1.1*** |
| Stress | 10.4±3.2 | 13.7±3.3 | 1.8±0.3*** | 8.6±2.6" |

n = 10 ♀/group
* = $P < 0.05$ Control vs. PG–$I_2$
*** = $P < 0.001$ Control vs. PG–$I_2$
" = $P < 0.05$ PG–$I_2$ vs. PG–$I_2$ + DNA Inhibitor

From the results the following conclusions have been drawn:

(1) DNA-synthesis inhibitors, CYAR and partly RUMY, inhibit the cytoprotection by PG–$I_2$, while DTIC was ineffective.

(2) In the indomethacin model RUMY strengthened the effect of PG–$I_2$. We have no explanation for this phenomenon.

(3) The results presented are in accordance with our previous results and raise the possibility that cytoprotection needs not only new protein synthesis but also new cell formation [3].

(4) The exact mechanism of action of DTIC is unknown. Therefore we have no explanation for its insignificant effect.

## Acknowledgement

This study was supported by the Hungarian Ministry of Health Research Grant TPB-EüM-46.

## REFERENCES

1. Bálint, G. A., Varró, V.: Antinomycin inhibits gastric mucosal protection by prostacyclin in rats. Prostaglandins **21,** 255–258 (1981)
2. Bálint, G. A., Varró, V.: Prostacyclin and proteinsynthesis inhibitors. Interaction on experimental gastric ulcer. Proc. 7th World Congress of Gastroenterology, Stockholm, 1982
3. Bálint, G. A., Varró, V.: Cytoprotective effect of prostacyclin and protein synthesis. Acta Physiol. Hung. **61,** 115–122 (1983)

*Acta Medica Hungarica, 41 (4), pp. 263—277 (1984)*

# INTESTINAL RESPONSE IN AGING: CHANGES IN RESERVE CAPACITY

L. PÉNZES

GERONTOLOGY CENTRE, SEMMELWEIS UNIVERSITY MEDICAL SCHOOL, 1085 BUDAPEST, SOMOGYI B. u. 33, HUNGARY

(Received July 18, 1983)

In contrast to the cardiovascular system, the gastrointenstinal tract in old age does not reveal the marked structural and functional deteriorations that might be expected to arise from the body's aging process. Besides preserving an active mass of functional tissue, the intestinal regulatory mechanisms show a precise and functional response to the changing circumstances of aging.

With regard to the aging processes, therefore, the relative physiological stability of the gastrointestinal system is very important. A possible cause of the maintenance of its functional capacity may be the processes involved in the cellular aging and rapid turnover of the gastrointestinal epithelium. This part of the gut remains practically unchanged and may continue its growth processes almost up to the point of death. Thus, intestinal functions do not seem to deteriorate even in late age. Kinetic studies indicate that the intestinal response in senescense to secure normal absorption processes occurs through an adaptation of transfer (carrier mediated) mechanisms. Such model studies have suggested that with age the affinity of the intestinal carrier mechanisms for the absorption of glucose and neutral amino acids are diminished. Appropriate changes in absorption occur i.e. in the young a faster rate of absorption is achieved at low luminal concentration than in the old. The amounts and activities of many essential intestinal enzymes have been observed to be reduced with age. This too may lead to a slower rate of absorption. In old age therefore it is the rate of intestinal absorption that undergoes a significant change rather than an overall deterioration in absorptive ability.

Our present knowledge indicates that there are at least two important regulatory mechanisms that enable the aged gastrointestinal tract to adapt itself to its altered functional capacity. On the one hand, the response of the intestinal epithelium mediated by the various rapid repair processes for the uptake of substances needed by the organism, and, on the other hand, the existence of the current load in the luminal environment controlling the intestinal metabolism and homeostasis in a normal way during aging.

An analysis of the close relationship that exists between structure and function in the gastrointestinal tract during aging, in particular the reasons for the observed alterations of the absorptive surfaces, will undoubtedly be useful in the practical elucidation of such problems as mucosal injuries, resection and malabsorption therapy.

**Keywords:** gastrointestinal tract, intestinal morphology, intestinal functions, absorption, aging.

## Introduction

Nowadays the increased average lifespan has created an urgent need to elucidate the mechanisms involved in the maintenance of a healthy old age. This can in part be achieved through a better and more thorough understanding of nutrition and associated metabolic activities in old age.

Send offprint requests to L. Pénzes H–1085 Budapest, Somogyi B. u. 33, Hungary

In the present report an attempt has been made to summarize the results of a 15-year survey of the effect of aging on the function of the small intestine. A study of changes in gastrointestinal physiology concerning aging *per se* rather than of the diseases occurring predominantly in the elderly was the main purpose of the present review. On the other hand, it will also discuss the information available on the changes related to geriatric subjects. Above all it will deal with the changes that occur in intestinal absorption during aging and attempt to explore the way in which the small intestine maintains its ability to function normally even in the extremes of old age. An effort was made to examine the ability of intestinal metabolism in old organisms to maintain homeostasis during aging by a variety of regulatory mechanisms, many of which can be observed in the small intestine almost up to the point of death. Although it is possible to state the time when the intestinal mucosa can be said to have reached maturity, it is a harder task to determine a precise point of time when aging can be said to have actually started. Still, some age-dependent malfunctions rarely occur within the intestinal tract and hence the establishment of biological aging in this part of the body cannot be determined unambiguously. Although precise and detailed observations have been reported [21] on the process of intestinal maturity, e.g. comparison of pre- and post-natal periods of development of the period of adaptation and the state of adult maturity, the results to be reported will mainly be confined to the changes connected with the process of senescence.

## *Morphological Changes*

### *Weight and size*

It is difficult accurately to quantify the changes that occur in the size of the gastrointestinal tract during senescence. According to Moog [31] the weight of the small intestine in SWR/J mice of both sexes increased continuously by about 60% during aging. This applied particularly to the terminal regions of the small intestine where mainly amyloid-like material was being accumulated in the *lamina propria* as age advanced. In addition, Moog was unable to find an increase in intestinal weight between female mice of 3 and 6 months of age in spite of their body weight gain during this period. However, between 6 and 12 months of age the intestinal weight increased to a greater relative extent than did body weight. In contrast to these observations, a slight increase, was only observed in the growth of the small intestine of Wistar rats which were older than 24 months of age; furthermore, there were no differences if the intestinal weight was related to body weight.

Information on the relative changes in size and length of the small intestine during aging is scarce. Our data [35] have shown that the absolute, unloaded small intestinal length of 6-month old, 12-month old adult and 27-

month old unpaired female Wistar rats remained virtually unchanged. At about half a decade earlier, Varga [44] found that the length of the small intestine of male Wistar rats increased considerably between 20 and 561 days of age. Using $^{85}Sr$-labelled microspheres, this author determined the effect of age on gastrointestinal motility in the rat, and concluded that although there was no correlation between gastric emptying and propulsive motility of the small intestine with age, the propulsive ability in the large intestine and coecum increased with age. On the other hand, it is well accepted that the majority of motility disorders in old age are due to disturbances associated with diabetes, cerebrovascular insults and possibly drug abuse. These conditions may influence either directly or indirectly the motor activity of the gastrointestinal tract [2].

## *Villi*

It is essential that consideration be given to the changes that occur with aging to the absorptive surfaces of the intestine, since they are the only one of the decisive factors which determine the rate of absorption. In the work of this laboratory [23] it was observed that, in the female rat, the height of the villi decreased with age. Reductions of the length of the villi were most conspicuous in the duodenum and ileum and less striking in the jejunum. Clarke [10] showed that during the first year of life there was a decrease in the height of the villi in the proximal regions of the small intestine of male rats. Also in male rats, Höhn et al. [20] observed that the mucosal atrophy was confined mainly to the proximal part of the intestine with the villi undergoing a considerable (approximately 25%) reduction in length. It is generally accepted that the number of villi remains relatively constant thoroughout life. As a result of a similar work, Clarke [9] was able to show in the male rat that the number of villi does not change significantly with age. Additionally, the villi should be considered as fixed functional units during life, and under extreme situations such as starvation, their size rather than their number is affected. Thus it may be concluded that over a complete lifespan there is no diminution in the number of villi. It must, however, be pointed out that in the proximal regions of the rat intestine fusion of villi becomes more frequent with age, and therefore a slight decrease in the number of villi may be observed in the later stages of life.

Other observations have been obtained using mice. Moog [31] emphasized that the villi underwent a significant increase in length, by up to nearly 20% in 24 month old SWR/J mice of both sexes when compared to 6-month old animals. Ecknauer et al. [15] have recently reported that the size of the proliferative units, the rate of cell production and the size of the functional compartment does not change significantly throughout life.

As there is no experimental evidence to show a decrease in cell loss from the intestine during aging, Ecknauer et al. [15] concluded from their findings that there must be an endogenous stimulus for the gastrointestinal tract to continue its growth almost up to the point of death. This growth is manifested through the formation, with age, of new villus-crypt units. Data on human subjects [47] support the findings obtained in rats; thus although the number of villi does not change during life, changes occur in their length and width, so that they become shorter and thicker with advancing age. The recent finding of Warren, Pepperman and Montgomery [46] that the mucosal surface is significantly reduced in 60–73 year old subjects tends to confirm this.

A certain amount of reconciliation between the contradictory reports mentioned above is clearly necessary. It seems highly probable that there is a large variation in the morphological changes observed to occur with age, not only between different species and sexes, but even within given strains. Throughout life these differences play a considerable part in the range of intestinal responses.

### *Regressive Changes*
### *Connective tissue and atrophy*

As has been pointed out [30] there is a notable absence in the literature of any reports of changes that occur in the intestinal wall and which may be lessened with age. The age-related chemical and intestinal structural changes that occur in the collagen and elastin of the intestinal connective tissue and smooth muscle elements only serve to accentuate the risks involved in taking biopsies at advanced age. These factors increase the importance of the various studies on the regressive changes that occur in the gut with age.

Suntzeff and Angeletti [43] using 3 month and 22 month-old CE-129 and Swiss mice of both sexes, showed that a fibrotic process takes place with aging in the *tunica propria* and results in a progressive replacement of the original stroma by a kind of fibrous sclerotic connective tissue. These changes begin after 1/3 of the animal's lifespan and progress further with age. Considerable pathological alterations were detected only in the terminal ileum of old SWR/J mice [31].

As has already been pointed out, the duodenal mucosa in senile (24–30 month old) male Wistar rats revealed conspicuous atrophy [20]. This atrophy would result in an approximately 25% decrease in the length of the duodenal villi. In contrast no appreciable differences were observed in the ileal region between adult (4 month-old) and senile rats. The authors claimed that the mucosal atrophy in the proximal small intestine was probably the result of a diminution in the rate of new epithelium formation. Using scanning electron-microscopy [21], it has been claimed that within the proximal small intestine of senile rats, not only were the villi different in length and width, but generally

smaller and revealed interspaces that were absent from 4 month-old rats. Again there were no notable changes in the distal regions of the small intestine.

### *Functional Alterations*

#### *Cellular turnover*

Senescence consists of a general deterioration process which takes place gradually and is accompanied by both structural and functional changes in the majority of organs and tissues. As the body grows older, different parts of the organism age at different rates and to different extents. As Lesher [29] formulated "for the most part, death is due to failure of an organ or organs with low cell turnover". It is generally believed that, under conditions of high cellular turnover, erroneous cells are eliminated from the proliferation compartment of the organ concerned [29]. The selection rate, however, does not work faultlessly. This is due on the one hand to a diminution of the synthetic processes associated with chromatin formation and, on the other hand, to the fact that cellular generation time increases with age.

In the small intestine of the $BCF_1$ mouse it has been shown that cellular proliferation is not linear with age. The greatest change occurs in the first instance at about 3 1/2 months when the growth of the animals begins to diminish and the period of young-adulthood starts. Following this there is little change until about the last third of the lifespan (825–1050 days of age), when a lengthening in generation time again becomes apparent.

The elevated intestinal cellular generation time observed in old $CAF_1$ mice provides the time necessary for the upward migration of cells from the depths of the crypts to the top of the villi (transit time). It was stated by Lesher [29] that the proliferative population decreased by more than one third in aged (825 day-old) $BCF_1$ mice. As pointed out by this author, the overall rate of cell production was decreased from about 10 cells/h/crypt in 100 day-old mice to 6 cells/h/crypt in 825 day-old mice. Thus according to Lesher, either the enterocytes must remain covering the villi for a longer time, or the cell population of the villi must be decreased if the covering remains constant. However, as the villus cell populations do not undergo any apparent change, the cellular survival time must be prolonged.

As has already been pointed out, this part of the intestine of the mouse becomes enlarged during aging. Much of this weight increase may be attributed to an increase in villous height which in turn extends considerably the size of the enzym-bearing surface. According to Moog [31] the slower transit times in old animals, at least in the $CAF_1$ strain, result in that the villous cells in the distal regions of the small bowel become richer in alkaline phosphatase, maltose and sucrose than those of the young animals as the concentrations of all these enzymes increase as the cells move upwards (Nordström et al., Moog and Grey, Moog et al. cit.: [33]).

Similar observations were made on the small intestine of the rat. The proximal region of the small gut is known to exhibit a marked reduction in villous length during aging whereas the distal portions are not reduced. Höhn et al. [20] found that the $^3$H-thymidine labelling index is about 30% in the proliferation compartment of the crypts of young-adult and old rats, indicating that the same proportion of cells are in the "S" phase of generation time in the indifferent zone of the small intestinal crypts in both groups of animals. This agrees with the findings of Lesher [29] concerning the duodenum of the mouse, with the addition that generation time in the crypts of old rats is decreased by about 35%. Thus atrophy of the duodenum in old animals is the consequence of a delayed generation time that occurs with age.

With regard to the colon, Hamilton [17] described that the changes in cellular repopulation observed after X-ray injury in young and old C57B1 mice were based on an overshot of the labelling index; changes in crypt size began later and continued longer in old than in young C57Bl mice. All results were limited by differences, with age, in the response of the gut to acute irradiation. The author sought to explain this by the fact that the feedback control mechanisms, which in young mice result in a rapid increase in cell production before returning to a normal labelling index soon after the crypts have become enlarged, are less precise in older animals. In old organisms, the control mechanisms generally need far more time in which to achieve any alteration in the proliferation rate.

### *Enzymes*

### *Alkaline and acid phosphatase*

Höhn, Gabbert and Wagner [20] found significant histochemical differences in alkaline and acid phosphatase activity of the intestinal mucosa between 4 month-old "adult" and 24–30 month-old senile rats. Nevertheless, densitometric and statistical evaluation of the changes of these two enzymes in the proximal regions of the small gut showed a considerable difference in their circadian rhythm and activities between 4 and 24 month-old rats; an apparent loss in activity was observed in the duodenum of senile rats. The authors were able to show that the cause of these changes in the aged rats was due primarily to villous atrophy as a result of the reduced number of enterocytes. The circadian rhythm in the activity of both enzymes was synchronous in younger and old animals, but there were marked differences in the circadian amplitudes; in the old animals the fluctuations were smaller. This calls attention to the limited time-dependent capacities of regulatory mechanism that exist in aging.

Sayeed and Blumenthal [40] reported on a reduction of up to 50% in the activity of alkaline phosphatase in 34 month old B6D2F$_1$/J mice. Some doubt must, however, be expressed concerning this observation due to the

methodology used. Moog [31] found that the activity of alkaline phosphatase was not decreased in 24 month old SWR/J mice. Expressing the total enzyme activity on a body weight basis shows that the aging body is in fact better supplied with the enzyme than is the young. As pointed out by the above author, the criticism of these studies is the inability to state what proportion of the total enzyme activity is associated with the membrane of the villous structure. Nevertheless, the main conclusion from these observations is that increased body and intestinal weight are associated proportionally with the increased length of villi which in turn results in a greater enzyme-bearing surface area.

### *Disaccharidases and other enzymes*

Rommel and Böhmer [37] observed that the activity of intestinal disaccharidases i.e. maltase, sucrase and lactase, was significantly lower in ageing rats (12 and 24 month old animals) than in 3 month old animals. Similar observations were made in humans; thus, in old American negroes low lactase levels occur frequently [48] but no age-dependent changes were noted in the activity of alpha-disaccharidases. In both white and black races, namely, age and race are far more decisive factors in the development of low intestinal disaccharidase levels than are mucosal injuries. On the other hand, in the baboon, lactase, cellobiase and alkaline phosphatase activities are highest in the young while diminished activity and changes in tissue distribution occur with advancing age. As in humans, there were no age-dependent differences in the activities of sucrase, maltase, palatinase and trehalase.

As in the case of the changes in alkaline phosphatase activities, Moog [31] found that the activities of maltase and sucrase did not decrease in aged SWR/J mice. In fact, when expressed per unit of body weight their activities were lower than those of the young control animals. These observations might well be explained by the increased height of the villi; on the other hand it was suggested by Moog that the mucosal surface of the 24 month-old mice was quite capable of splitting maltose and sucrose. In fact, maltase and sucrase activities, when expressed per unit of body weight, proved to be about 30% higher in old than in young mice. Observations on intestinal phosphomonoesterase activity [39] have shown that although $V_{max}$ values were lower in 34 month old mice, the Michaelis constant ($K_m$) was unaffected. This therefore suggests that as compared to 11 months old young-adult mice, in old mice concentration of the enzyme was reduced but its structure remained relatively unchanged.

### *Absorption*

The question whether absorption from the intestine changes during aging is difficult to answer. Most studies were done in rodents and often their

age has not been recorded. In most cases they were merely considered as to be young, adult and just old. Most frequently the experiments have been performed up to adulthood but have not been extended to include the period of senescence. As a result of the physiological changes that have been observed to occur in intestinal absorption only cover a given period of ontogeny that excludes aging *per se*.

Similarly, the findings in human subjects are also difficult to evaluate. Wirts [49] described that the human gastrointestinal system undergoes basic alterations with aging. Thus atrophic gastritis seems to appear with considerable frequency from the 3rd decade onwards. The changes which gradually occur in the gastrointestinal tract cover such alterations as decrease of mucosal thickness, increase of leukocyte infiltration and lymphoid aggregates as well as pyloric heterotopy. All these changes could sometimes lead to achlorhydria. Bowman and Rosenberg [5] suggest that the prevalence of achlorhydria in the elderly is uncertain, but there is a report according to which nearly 70% of 657 consecutive patients of a geriatric unit were achlorhydric [4]. Nevertheless, this may be the only aspect of physiological decline in some intestinal functions that frequently manifests itself with aging. There are almost no indications of any occurrence of a so-called wear and tear process in the aged gastrointestinal tract similar to that which characterises many other parts of the body, e.g. the cardiovascular system.

It must be added that because of the extreme variation that exists in the way the data are expressed, any attempt to compare the results found in the literature is difficult, as also is the ability to find any possible correlation between aging and the supposed reduction in intestinal absorption.

### *Sugars*

Results reported in the literature mainly concerned the absorption of sugars and calcium. Among the sugars, the absorption of xylose has received most attention. Kendall [25] stressed that aging *per se* is the decisive factor in the evaluation of xylose absorption tests. The decreased excretion of xylose that occurs with age does not indicate a faulty absorption but points to impaired renal function. Rommel and Böhmer [37] suggested that in the aging organism an alteration occurs in the metabolism of xylose. It has also been shown [16] that the reduced sugar absorption displayed by the aged results from either an alteration in the efficiency of the transport systems involved or a reduction of the mesenterial blood supply; as a result of the latter, there would also be a restriction of oxygen supply to the metabolically very active intestinal tissue. On the other hand, Varró et al. [45] pointed out that the intestinal tract possesses a well-developed regulatory capacity; there would have to be a serious deterioration in the mesenterial blood supply before any significant reduction would occur in either the intestinal transport of glucose or oxygen

consumption. On the evidence of Dardik et al. [11] it is suggested that sclerotic stenosis of mesenteric and coeliac arteries may cause not only intestinal angina but also malabsorption. Such vascular changes may also be the cause of the reduced xylose absorption that occurs in the aged. However, Rosin et al. [38] have shown that due to the extreme variation displayed by existing data, the xylose test cannot be considered accurate and its value in the evaluation of data is open to doubt. It is therefore not surprising that the ability to assess the various criteria involved in the changes of intestinal absorption that occur during aging is fraught with difficulties. Verification of this seems to be provided by the observations of Webster and Leeming (cit. [33]) who found that only 26% of all geriatric subjects investigated showed a reduced xylose absorption.

It must also be said that the results from animal experiments are no easier to interpret. Thus Klimas [26] who performed absorption studies in 2, 5, 10, 15, 20, 26 and 32 month old rats, reported that, providing it was expressed as mg/h or mg/cm intestine/h, the rate of absorption increased for the first 1/3 of the rat's lifespan. Following this period, there was no further change. The rate of adsorption in the senescent animals was constant. If absorption was expressed as mg/100 g bw/h, then it was found to diminish during the first 1/3 of the lifespan after which time it became constant. This should be interpreted in the light of the author's further observations that the weight and length of the small intestine as well as body weight, continuously increase in the rat, a feature which is most conspicuous during the first five months of life.

In previous investigations from this laboratory [34] a kinetic approach was used to study the age-dependent absorption of d-glucose. From the results obtained it was clear that the Michaelis constant ($K_m$) of glucose absorption increased almost linearly with age in 6, 12 and $\geqslant$ 24 month-old female Wistar rats. The Michaelis constant was considered a measure of affinity of the intestinal carrier system for the molecules that were absorbed from the lumen. This evaluation was chosen because the dose dependent absorption of d-glucose, and of amino acids, proved to be a rate-limiting process possessing kinetics that approximated the Michaelis–Menten scheme. The results showed a parallel decline with age of carrier affinity. This indicated that transport from the intestinal lumen of sugar molecules at low (initial) concentration took place at a higher rate in the young than at advanced age. Thus a possible role in absorption for age-dependent changes the intestinal glucose carriers cannot be ignored. It must be added that the occurrence of low activities of intestinal enzymes are not uncommon in the aged small intestine and therefore the ability to absorb low concentrations of substrates should be taken into account. As a result of the above, slow absorption is to be expected in old individuals. Recent data from this laboratory [23] have indicated that the

reduction of absorption low intestinal concentrations (4 mM) of d-glucose in the 24 month-old rat may also be due to a decrease in length of the villi.

### *Amino acids*

Studies of amino acid absorption in the elderly have rarely been the subject of gerontologists. Observations of Pénzes [34] on the absorption of amino acids from the small intestine of old rats have shown the following. As with glucose, the kinetic parameters, i.e. $K_m$ and $V_{max}$ for glycine, alanine, valine, leucine, phenylalanine, tryptophan and proline were generally higher in $\gg 24$ month-old than in 6 month-old rats. This was indicative that in old age whilst the affinities of these amino acids for the intestinal carriers was less, there was simultaneously a reduced rate of transport at low luminal concentrations. The absorption of basic amino acids (lysine, arginine and histidine) was found to be different; aged rats displayed higher intestinal affinity (lower $K_m$ values) than did the young. The reason for this must lie in the fact that the basic amino acid components are low in protein hydrolysates thereby providing them with a better competitive capability for transport through the villous membrane in comparison to the neutral amino acids, however, were there a competition for absorption of a protein meal, the molar ratios between rapidly transported and slowly transported amino acids would display a wide variation with time and over different segments of the small intestine.

### *Fats*

Becker et al. [1] observed that in aged, over 75 years old, humans there was a conspicuous delay in the absorption of unhydrolysated fats (hyperchylomicronaemia). The main cause of this was a diminished lipase synthesis. Thus, when appropriate of lipase were added to the diets in combination, with adequate quantities of fat, the "chylomicron curves" determined became similar to those obtained in 18-year-old subjects. It has been pointed out by Citi and Salvini [8] that absorption is affector at the mucosal level to which the reduced number of enterocytes may contribute. On the other hand, reductions in intestinal absorption may probably be associated with low levels of pancreatic lipase. This seems to be confirmed by Pelz, Goffried and Sooes [32] who were able to observe that faecal fat was high in 17 of 43 institutionalized elderly people, whilst faecal free fatty acids were low in 10 of the 17 subjects. Above all, balance studies on the effect of dietary fibre on energy utilization seem to support the idea that age does not considerably influence the apparent digestibility of fat [5].

### *Electrolytes*

That the absorption of calcium diminishes with age is well proven. Using the everted sac technique, Schachter et al. [41] showed that calcium transport

in adult (12 month-old) rats was less than in 1–4 month-old rats. On the other hand, Schachter et al. [42] pointed out that the oxygen-dependent calcium accumulation during the transfer process was not as evident in old as in young rats, as the intestinal uptake of calcium in the aged is strictly associated with a passive diffusion.

The excretion rate of endogenous calcium does not necessarily relate to calcium balance. In a series of elegant studies, Hironaka et al. [18] found that the calcium excretion of old (22–23 month) rats, mainly through faecal loss, was significantly higher than that of adult (10–12 month-old) rats in spite of the fact that the old rats were in calcium equilibrium throughout the 18-day experimental period. It was claimed that the aged animals were able to compensate their apparent greater calcium loss by an increased calcium uptake from the food. The greater loss of endogenous calcium by the aged rats was caused by a smaller retention of skeletal calcium. Credence for these conclusions is provided by the known increase in transit time of food in the gastrointestinal tract of old animal and the changes that occur in the feeding habits. The biological half life time of plasma calcium was $12.4 \pm 1.0$ days in the aged as compared to $11.4 \pm 1.0$ days in adults ($P > 0.05$). Such an observation was not that expected as the effect of an increased calcium absorption in the old would be to diminish the half life of plasma calcium. The only possible explanation is that continuous labelling of plasma calcium occurred through $^{45}Ca$-exchange with the skeleton. Since the old rats would have lost less skeletal calcium, the specific activity of the plasma calcium would have remained.

From the above the authors concluded that through an enhanced intestinal absorption the aged rat is able to adapt itself to the greater calcium requirement that accompanies old age. There is an associated increased skeletal absorption. Since the aged rat is capable of compensating a greater endogenous calcium loss through augmented intestinal absorption, calcium equilibrium can theoretically be maintained in the old as in the young. A clear analogy exists between this situation in the old and the greater calcium absorption that occurs in the young when there is an intensive demand for bone formation.

In view of the differences that have been noted to exist in the intestinal absorption of calcium between 6 month and 24 month-old rats, an attempt has been made to promote intestinal calcium absorption in the aged rat by the addition to the diet of l-lysine [36]. It was found in 40 minute and 24 hour studies *in vivo* that the addition of l-lysine significantly increased the intestinal calcium uptake in the aged animals. As any disturbance of calcium metabolism in the old has an effect on a number of (mainly endocrinological) factors, the possibility exists that an improvement of intestinal calcium absorption may have therapeutic benefits.

The observations that have been made on human subjects are not only few in number but also somewhat controversial in their content (see Avioli

et al., Bullamore et al. and Ireland and Fordtran, cit. Pénzes [33]. Avioli et al. showed that calcium absorption in elderly women was lower than in premenopausal subjects. Similar observations were made by Bullamore et al. who were able to demonstrate a clear decline in the absorption of calcium in both male and female subjects over the age of 60. Similarly, Ireland and Fordtran found that more calcium was absorbed in adults than in subjects over sixty years of age. Using a kinetic approach the authors concluded that, as a result mainly of differences in the activity of intestinal transfer systems, there was a marked difference in adaptive ability between aged and young subjects. Bogdanoff et al. (cit. [33]) but concluded that aged (>65 years) male subjects did not absorb less calcium than young subjects, while it was suggested by Alevizaki et al. (cit. [33]) that calcium absorption decreased exponentially from the 12th year of age onwards, probably as a result of the age-dependent deterioration of intestinal function. More recent data [28] have again indicated that calcium utilization in the 60–80 year age group is considerably less than in 35 year old subjects.

According to Draper [14] magnesium absorption does not change with age. Similarly, studies using $^{24}Na$ and $^{42}K$ have indicated that there are no apparent changes in sodium and potassium absorption in rats between the ages of 5 and 34 months [27].

Reports on the absorption of iron in old age are scarce. Studies in humans [22] showed that absorption of inorganic iron was less efficient in the elderly than in the young. This could however not be related to any change in haem-bound iron. Yeh et al. [50] using rats of different ages, observed that absorption of radioactive iron decreased from the 20th month onward. The conclusions were based not solely on faecal excretions, but included measurements of tissue incorporation and specific activities of blood. It should be pointed out that one of the most frequently occurring abnormalities of aging i.e. achlorhydria, may be related directly to the development of hypochromic microcytic anaemia through a diminished supply of gastric acid which in turn leads to a reduction of iron absorption [49].

### *Vitamins*

Numerous early reports were concerned with the absorption of vitamins in senescence. The studies of Rafsky and Newman (cit. [33]) showed that vitamin A uptake in 69–89 year-old subjects was less than in young subjects. Yiengst and Shock, and Shock (cit. [33]) were unable to find differences in vitamin A tolerance caused by age *per se*. Nevertheless, Yiengst and Shock emphasized that there may be a delay in vitamin A absorption in persons over the age of 70. In later work with rats vitamin A absorption from the small intestine was found to increase linearly from 15 month up to the 39 month of

age (Hollander and Morgan; cit. Bowman and Rosenberg, 5). Using radiocarbon labelled thiamine, Draper [13] showed that absorption in 20–24 month-old rats was some 75% whereas in 19–20 month-old rats it was as high as 90%. Urinary thiamine excretion in 22 senescent rats (Rafsky and Neuman cit. [33]) indicated that significantly less thiamine was absorbed in the old than in the young animals. This finding was confirmed later in humans (Mills' cit. [33]). On the other hand, Kirk and Chieffy (cit. [33]) observed that thiamine absorption changed only slightly with age. The data of Thompson (cit. [5]) showed no significant difference in thiamin excretion between 80 year-old and young people. Simultaneously, from experiments involving human subjects ranging in age from 3 months to 40 years, Jusko et al. [24] concluded that intestinal transport of riboflavin was considerably quicker in the young than in the aged; it was also noted that renal excretion of riboflavin did not change over the age range studied.

There does not appear to be any age effect in the absorption of folic acid [3]. Vitamin $B_{12}$ absorption in senescence also appears to remain unchanged [6, 7, 12]. It was, however, suggested that the situation depended much upon faecal excretion and hepatic function. Under normal circumstances, in 75–90 year-old subjects sufficient intrinsic factor is secreted by the gastric wall in order to ensure adequate absorption of vitamin $B_{12}$ from the ileum. Plasma $B_{12}$ levels have shown [6, 7] that they underwent a continuous reduction between 6 months of age and senescence, probably as a result of the reduced intestinal uptake. Observation in 20 to 92 year old human subjects by Gaffney et al. (cit. [33]) showed no differences in vitamin $B_{12}$ absorption. These findings are of clinical interest because in geriatric subjects with achlorhydria or on antacid or cimetidine therapy less vitamin $B_{12}$ may be absorbed. Under such conditions, oral cyanocobalamine may be helpful [5].

From data concerning human subjects above the age of 75 years it has been concluded by Chieffy and Kirk (cit. [33]) that absorption of vitamin C does not differ from that of young adults. As regards vitamin $D_3$, Holt and Dominguez [19] found a reduced intestinal uptake in aged rats, but Bowman and Rosenberg [5] quote Hodkinson et al. and concluded that it was appropriate to use the term "osteomalacia of the housebound" which precisely describes the situation of the elderly concerned.

## REFERENCES

1. Becker, G. H., Meyer, J., Necheles, H.: Fat absorption in young and old age. Gastroenterology, **14,** 80–90 (1950)
2. Berges, W., Erckenbrecht, J., Wienbeck, M.: Motilitätsstörungen des Verdauungstraktes im Alter. Z. Gerontol. **15,** 102–106 (1982)
3. Bhantumnavin, K., Wright, J. R., Halsted, C. H.: Intestinal transport of tritiated folic acid ($^3$H–PGA) in the everted gut sac of different aged rats. Johns Hopkins Med. J. **135,** 152–160 (1974)

4. Bird, T., Hall, M. R. P., Schade, R. O. K.: Gastric histology and its relation to anaemia in the elderly. Gerontology (Basel), **23,** 309–321 (1977)
5. Bowman, B. B., Rosenberg, I. H.: Digestive function and aging. Hum. Nutr. Clin. Nutr. **37C,** 75–89 (1983)
6. Chow, B. F., Gilbert, J. P., Okuda, K., Rosenblum, C.: The urinary excretion test for absorption of vitamin $B_{12}$. 1. Reproducibility of results and agewise variation. Am. J. Clin. Nutr. **4,** 142–146 (1956)
7. Chow, B. F., Wood, R., Horonick, A., Okuda, K.: Agewise variation of vitamin $B_{12}$ serum levels. J. Geront. **11,** 142–146 (1956)
8. Citi, S., Salvini, L.: The intestinal absorption of $^{131}$I-labelled olein and triolein, of $^{58}$Co-vitamin $B_{12}$ and $^{59}$Fe in aged subjects. G. Geront. **12,** 123–126 (1969)
9. Clarke, R. M.: The effect of growth and of fasting on the number of villi and crypts in the small intestine of albino rats. J. Anat. **112,** 27–33 (1972)
10. Clarke, R. M.: Effects of age on mucosal morphological and epithelial cell production in rat small intestine. J. Anat. **123,** 805–811 (1977)
11. Dardik, H., Seidenberg, B., Parker, J. G., Hurwitt, E. S.: Intestinal angina with malabsorption treated by elective revascularization. JAMA, **194,** 1206–1210 (1965)
12. Davis, R. L., Lawton, A. H., Prouty, R., Chow, B. F.: The absorption of oral vitamin $B_{12}$ in an aged population. J. Geron. **20,** 169–172 (1965)
13. Draper, H. H.: Physiological aspects of aging. I. Efficiency of absorption and phosphorylation of radiothiamine. Proc. Soc. Exp. Biol. Med. **97,** 121–124 (1958)
14. Draper, H. H.: Physiological aspects of aging. V. Calcium and magnesium metabolism in senescent mice. J. Nutr. **83,** 65–72 (1964)
15. Ecknauer, R., Vadakel, T., Wepler, R.: Intestinal morphology and cell production rate in aging rats. J. Geront. **37,** 151–155 (1982)
16. Geokas, M. C., Haverback, B. J.: The aging gastrointestinal tract. Am. J. Surg. **117,** 881–892 (1969)
17. Hamilton, E.: Cell proliferation and aging in mouse colon. Cell Tissue Kinet. **11,** 423–431 (1978)
18. Hironaka, R., Draper, H. H., Kastelic, J.: Physiological aspects of ageing. III. The influence of ageing on calcium metabolism in rats. J. Nutr. **71,** 346–360 (1960)
19. Holt, P. R., Dominguez, A. A.: Intestinal absorption of triglyceride and vitamin $D_3$ in aged and young rats. Dig. Dis. Sci. **26,** 1109–1115 (1981)
20. Höhn, P., Gabbert, H., Wagner, R.: Differentiation and aging of the rat intestinal mucosa. II. Morphological, enzyme-histochemical and disc electrophoretic aspects of the aging of the small intestinal mucosa. Mech. Age. Devel. **7,** 217–226 (1978)
21. Höhn, P., Schäfer, A., Gabbert, H.: Differentiation and aging of the intestinal mucosa of the rat. I. Morphological, enzyme-histochemical and disc electrophoretic aspects of the prenatal and postnatal differentiation of the intestinal mucosa. Mech. Age. Devel. **6,** 35–51 (1977)
22. Jacobs, A. M., Owen, G. M.: The effect of age on iron absorption. J. Geront. **24,** 95–96 (1969)
23. Jakab, L., Pénzes, L.: Relationship between glucose absorption and villus height in aging. Experientia (Basel) **37,** 740–741 (1981)
24. Jusko, W. L., Levy, G., Yaffe, S. J.: Effect of age on intestinal absorption of riboflavin in humans. J. Pharm. Sci. **59,** 487–490 (1970)
25. Kendall, M. J.: The influence of age on the xylose absorption test. Gut **11,** 498–501 (1970)
26. Klimas, J. E.: Intestinal glucose absorption during the life-span of a colony of rats. J. Geront. **23,** 529–532 (1968)
27. Korbuly, D.: Note on the influence of age on sodium and potassium absorption from the intestine of the rat. Gerontologia, (Basel) **12,** 99–105 (1966)
28. Lender, M., Verner, E., Stankiewicz, H., Menczel, J.: Intestinal absorption of $^{47}$Ca in elderly patients with osteoporosis, Paget's disease and osteomalacia. Effects of calcitonin, oestrogen and vitamin $D_2$. Gerontology, (Basel) **23,** 31–36 (1977)
29. Lesher, S.: Chronic irradiation and ageing in mice and rats. Radiation and Ageing. Proc. Coll. Semmering, Austria 1966, p. 183–206.
30. Linaker, B. D.: Ageing and the small intestine. Lancet **2,** 993 (1978)
31. Moog, F.: The small intestine in old mice. Growth, alkaline phosphatase and dissacharidase activities, and deposition of amyloid. Exp. Geront. **12,** 223–235 (1977)
32. Pelz, K. S., Goffried, S. P., Sooes, E.: Intestinal absorption studies in the aged. Geriatrics **23,** 149–153 (1968)
33. Pénzes, L.: The intestinal absorption during reproduction and aging. D. Sc. Thesis, Budapest (1977)

34. Pénzes, L.: Intestinal absorption in the aged. Acta Med. Acad. Sci. Hung. **37,** 203–209 (1980)
35. Pénzes, L.: Letter to the Editor. Exp. Geront. **17,** 243–244 (1982)
36. Pénzes, L., Ádám, A., Boross, M.: Effect of l-lysine on the intestinal absorption of radiocalcium in ageing rats. Akt. Geront. **3,** 531–535 (1973)
37. Rommel, K., Böhmer, R.: Beziehungen zwischen Lebensalter, intestinalen Disaccharidasen und Monosaccharidabsorption der Ratte. Ärztl. Forschg. **26,** 453–457 (1972)
38. Rosin, A. J., Warin, J., Silk, D.: Evaluation of the d-xylose absorption test in elderly inpatients. Geront. Clin. **12,** 347–350 (1970)
39. Sayeed, M.: Age related changes in intestinal phosphomonoesterases. Fed. Proc. **26,** 259 (1967)
40. Sayeed, M., Blumenthal, H. T.: Age difference in the intestinal phosphomonoesterase activity of mice. Proc. Soc. Exp. Biol. Med. **129,** 1–3 (1968)
41. Schachter, D., Dowdle, E. B., Schenker, H.: Accumulation of $^{45}Ca$ by slices of the small intestine. Am. J. Physiol. **198,** 275–279 (1960)
42. Schachter, D., Dowdle, E. B., Schenker, H.: Active transport of calcium by the small intestine of the rat. Am. J. Physiol. **198,** 263–268 (1960)
43. Suntzeff, V., Angeletti, P.: Histological and histochemical changes in intestines of mice with aging. J. Geront. **16,** 225–229 (1961)
44. Varga, F.: Transit time changes with age in the gastrointestinal tract of the rat. Digestion **14,** 319–324 (1976)
45. Varró, V., Blahó, G., Csernay, L., Jung, I., Szarvas, F.: Effects of decreased local circulation of the absorption capacity of a small intestine loop in the dog. Am. J. Digest. Dis. **10,** 170–177 (1965)
46. Warren, P. M., Pepperman, M. A., Montgomery, R. D.: Age-changes in small intestinal mucosa. Lancet, **2,** 849–850 (1978)
47. Webster, S. G. P., Leeming, J. T.: The appearance of the small bowel mucosa in old age. Age Aging, **4,** 168–174 (1975)
48. Welsh, J. D., Poley, J. R., Bhatia, M., Stevenson, D. E.: Intestinal disaccharidase activities in relation to age, race, and mucosal damage. Gastroenterology, **75,** 847–855 (1978)
49. Wirts, W. C.: The aging intestinal tract: esophagus, stomach and small bowel. J. Am. Geriat. Soc. **9,** 933–939 (1961)
50. Yeh, S. D. J., Soltz, W., Chow, B. F.: The effect of age on iron absorption in rats. J. Geront. **20,** 177–180 (1965)

*Acta Medica Hungarica, 41 (4), pp. 279—285 (1984)*

*Nephrology*

# STATISTICAL ANALYSIS OF MONITORING EXAMINATIONS IN CHRONIC PERITONEAL DIALYSIS PROGRAM

É. A. Balás,[1] I. Taraba[2]

[1]DATA CENTER OF SEMMELWEIS UNIVERSITY MEDICAL SCHOOL; AND [2]NATIONAL INSTITUTE OF INTERNAL MEDICINE, BUDAPEST, HUNGARY

(Received March 10, 1983)

In dialysis centres the major part of laboratory tests is indicated by the routine monitoring strategy of the centre and not by the evidence of a new complication to be diagnosed. The aim of our study was to define an improved strategy, minimizing financial costs without loss of information. Findings of routine laboratory check-up of eleven patients undergoing chronic intermittent peritoneal dialysis were analysed. Using univariate and multivariate functions a considerable part of the laboratory test results can be estimated or predicted. From pre-dialysis measurements on serum the post-dialysis serum creatinine urea and uric acid levels can be predicted. The serum level of protein and hemoglobin can be estimated using the results of previous tests (performed maximum 7 weeks earlier) and current measurements on dialysate.

**Keywords:** peritoneal dialysis, test selection, statistical prediction

## Introduction

Management of patients on chronic peritoneal dialysis requires continuous follow up of serum parameters. It is, however, essential to bear in mind the limitations of periodic blood sampling (danger of viral hepatitis, anaemia, costs etc.). Considering the fact that the majority of laboratory tests serve periodic control, a design of blood sampling protocol should give a reasonable compromise.

Our purpose was to determine the appropriate laboratory testing strategy and to reduce the number of investigations.

## Materials and methods

The results of a battery of biochemical measurements were studied in 200 peritoneal dialyses of eleven patients undergoing chronic intermittent peritoneal dialysis (IPD) (Table I). No discrimination was made in the selection of patients for the study as to sex, diagnosis or previous blood chemistry findings.

Send offprint requests to Balás É. A., Bartók B. út 18, H-1111 Budapest, Hungary

The usual technique of IPD was to introduce 1500–2000 ml dialysis fluid (Peridisol I) into the peritoneal cavity and leaving it for 30 minutes equilibration time; afterwards the fluid was drain out. This process was repeated during 10 hours, 3 times a week. Creatinine was measured according to Jaffe, uric acid according to Carol, urea by a kinetic urease method. All methods were modified for the CentrifiChem 500 system. Ions (Na, K, Ca, P) were measured by flame photometry. Protein was estimated by the biuret reaction and haemoglobin by the method of Drabkin (Hemisol reagent).

If two laboratory tests are related by the fact that the result to be given by one of them can be determined from the result of the other, the first test is a function of the second. In mathematical sense the object is to determine the "best" function to estimate the value of

**Table I**

*Laboratory protocol and characteristics of the sample. Abbreviations in brackets are used in equations*

| Sample | Test | $\bar{x}$ | SD | |
|---|---|---|---|---|
| Pre-dialysis serum | Urea (su) | 14.7 | 3.5 | mmol/l |
| | Creatinine (sc) | 935 | 255 | μmol/l |
| | Uric acid (sa) | 680 | 147 | μmol/l |
| | Na (sn) | 140.1 | 2.8 | mmol/l |
| | K (sk) | 4.89 | 0.81 | mmol/l |
| | Ca (sca) | 2.07 | 0.2 | mmol/l |
| | P (sp) | 2.84 | 0.62 | mmol/l |
| Post-dialysis serum | Urea | 12.3 | 3.5 | mmol/l |
| | Creatinine | 793 | 214 | μmol/l |
| | Uric acid | 585 | 151 | μmol/l |
| | Protein (spr) | 49.8 | 7.2 | g/l |
| | Hemoglobin (sh) | 5.67 | 0.91 | mmol/l |
| | White cells | 5.38 | 1.48 | g/l |
| Dialysate | Urea (du) | 9.3 | 2.3 | mmol/l |
| | Creatinine (dc) | 604 | 195 | μmol/l |
| | Uric acid (da) | 356 | 108 | μmol/l |
| | Protein (dpr) | 1.18 | 0.72 | g/l |

a variable to be predicted (Y), corresponding to the predictor variables ($X_1, \ldots, X_N$). There are various ways in which the concept "best" can be defined mathematically but we used the least squares technique to minimize the average squared distance between predicted and measured values. The coefficient of multiple correlation was also calculated to assess the degree of the relationship between the predicted and measured results.

The estimate of dispersion about the fitted function (DF) may be written in notational form as follows

$$DF = \sqrt{\frac{\sum_{i=1}^{n} (y_i - y_p)^2}{n - 1}} = \sqrt{1 - r^2} \cdot S.D.$$

where $y_i$ is the measured and $y_p$ the corresponding predicted value of Y. S.D. denotes the standard deviation of Y, r is the coefficient of correlation and n the sample size. Both DF and r may be considered as measures of prediction accuracy.

Analysing the bivariate scatter plots we could conclude that the linear model was appropriate. In our work not only univariate functions were calculated and analysed but multivariate linear functions as well, to improve the predicting ability by including more predictor variables.

Considering the fact that it is impossible to reduce DF (or to increase the multiple correlation coefficient) by deleting predictor variables, the use of all possible predictors seems to be appropriate. Some predictors, however, increase r only slightly but complicate calculations markedly. For practical purposes (equations are given in the discussion) the best subset of predictors was identified by the leaps and bounds algorithm [2] deleting variables improving the prediction of Y nonsignificantly.

## Results

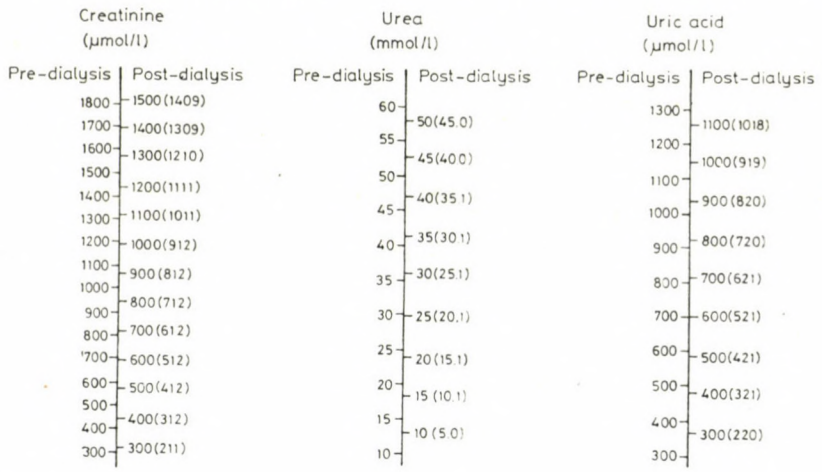
For estimation of the post-dialysis serum creatinine level the accuracy of the proposed mathematical representation of prediction can be seen in Fig. 1 where the least-squares line and corresponding equation are demonstrated. We estimated also the limits within which the true post-dialysis creatinine level is likely to lie (with 0.8 probability in Fig. 1).

On the basis of these results, in Table II a graphical aid is given for determining conditional expected value of postdialysis serum levels and the lower limit of confidence intervals. (The coefficient of correlation is 0.87 for urea and 0.91 for uric acid.)

In view of the poor correlation, blood urea (0.55) and uric acid (0.42) cannot be calculated accurately from the measurements of dialysates even by multivariate methods. (The estimate of dispersion about the fitted function [DF] is 6.12 mmol/l for urea and 12.8 $\mu$mol/l for uric acid.)

**Table II**

*Nomograms for estimation of post-dialysis serum levels.*
*The probability that the serum level higher than the value in brackets is 0.9*




The correlation of predicted and measured levels is close only for creatinine (0.86) but Lambrey et al. [4] showed that the biochemical data of residual peritoneal fluid (RPF) are suitable for the determination of blood levels of small molecules.

Unfortunately, plasma protein and haemoglobin can be accurately calculated neither from RPF, nor from the dialysate. Repeated blood tests cannot be avoided. To define the optimized blood sampling protocol, the results of the tests were analysed in comparison with the results measured 1,2, . . ., 7

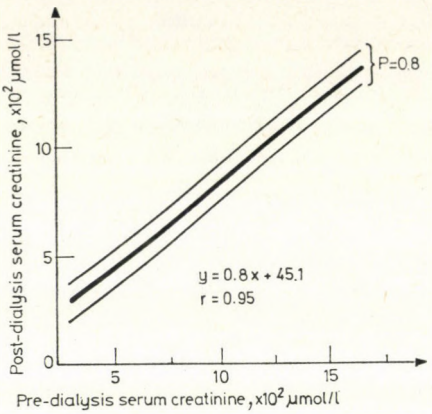



*Fig. 1.* **Regression equation and confidence band for prediction of the post-dialysis serum creatinine level. Regression line: y = 0.8 × +45.1; r = 0.95**

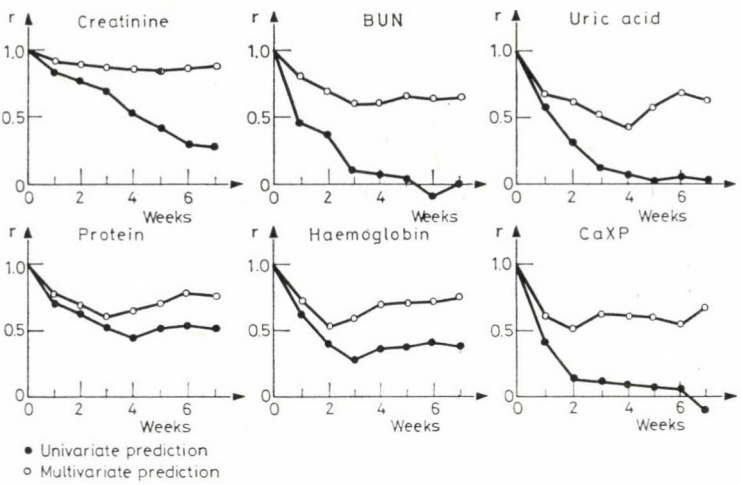



*Fig. 2.* **Time series of prediction success**

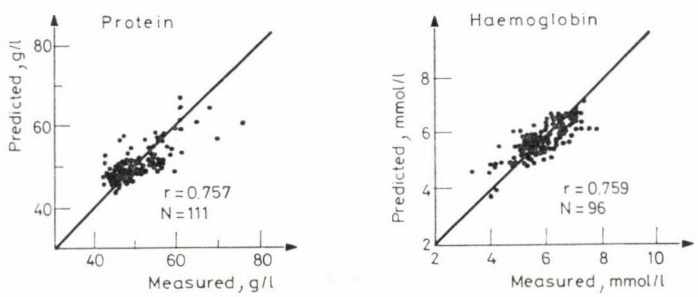



*Fig. 3.* **Multivariate prediction for 7th week**

weeks later. The number of such test pairs exceeded 100 for every space of time. Fig. 2 shows the rapidly decreasing correlation between "0 day" measurements and later results, as a function of time.

Another approach is to use the results of previous blood tests and current measurements of the dialysate. It is assumed that we ought to be able to improve our predicting ability by a combination of previous invasive and actual noninvasive tests. As a matter of fact the success of multivariate prediction always surpasses the results of univariate methods.

The correlation of predicted and measured serum levels of protein exceeds 0.5. The situation is similar with haemoglobin where the lowest correlation in the second week may be explained by the temporary modifying effect of blood transfusion.

Figure 3 presents the scatter diagrams of the predicted and measured values for protein and haemoglobin, where the accuracy of multivariate prediction for 7th week can be studied.

## Discussion

In uraemic patients on IPD the optimal timing of sampling is a natural requirement. Our optimality criterion was that an informative laboratory measurement is required only if its result cannot be estimated on the basis of the result of some other, previous tests.

The univariate prediction based on the preceeding value of the same parameter is accurate only in the first week. Later, with the exception of creatinine, the calculated coefficient of correlation is lower than 0.5. This means that for most tests the measured serum level alone is unacceptable or misleading as a predictor for more than two weeks.

Except for creatinine, the accuracy of multivariate prediction (i.e. the strength of the relationship between measured and predicted values) is not enough to establish the identity of these methods but it may be concluded that multivariate mathematical prediction is suitable for orientation.

For practical purposes the multivariate estimations, which are functions of all possible predictors, can be simplified by neglecting unimportant variables in the equation, as it has been discussed above.

Our suggestion for protein estimation in the 4th and 6th weeks is

$$spr_4 = 11.74 + 0.23 \cdot su_0 + 0.3 \cdot spr_0 + 3.64 \cdot sca_0 + 1.39 \cdot f_4 - \\ -1.01 \cdot dpr_4 - 0.01 \cdot da_4 + 0.28 \cdot du_4$$

$$spr_6 = 10.12 + 0.35 \cdot spr_0 + 6.12 \cdot sca_0 - 0.82 \cdot sh_0 + 1.08 \cdot f_6 + \\ + 0.38 \cdot du_6 - 0.02 \cdot da_6$$

where the index denotes the relative time of measurements (weeks), and f the removed excess fluid in liters. Other notations are defined in Table I. The

coefficient of correlation between measured and predicted value is 0.59 and 0.75, respectively.

The corresponding equations for haemoglobin are

$$sh_4 = 4.43 - 0.04 \cdot spr_0 + 0.41 \cdot sp_0 + 0.34sh_0\ 0.003 \cdot da_4 - 0.04 \cdot du_5$$

$$sh_6 = 4.05 - 0.05 \cdot spr_0 + 0.37 \cdot sk_0 + 0.37sh_0 + 0.003 \cdot da_6 - 0.05 \cdot du_6$$

The coefficients of correlation are 0.65 and 0.66.

Estimation of a single linear function which contains also the time elapsed since the last invasive measurement as a predicting function, is inappropriate because of obvious nonrandomness (i.e. time dependent growing tendency) of errors.

Lambrey et al. [4] have documented the usefulness of laboratory measurements of RPF in the estimation of serum levels. There was no significant difference between RPF and plasma values for urea, creatinine, sodium, potassium and phosphate. The correlation between measurements of RPF and plasma was higher than 0.8 for Ca, but the plasma protein cannot be calculated reliably from the protein concentration of RPF because of the poor correlation (0.4).

The present evidence suggests that measurement of creatinine in the dialysate is suitable for the estimation of its level in the dialysate serum level and this might be useful if we have not enough RPF. Predialysis serum creatinine can be estimated from its level in the dialysate by the following equation.

$$sc = 250.34 + 45.83 \cdot dpr + 7.07 \cdot du + 1.02 \cdot dc - 0.57 \cdot da + \\ + 14.87 \cdot s - 0.27 \cdot t$$

where s is the dialysis solution in liters and t the duration of dialysis in minutes. The coefficient of correlation is 0.86.

## Conclusions

The above results suggest that our standardized IPD technique has a calculable short and long-term effect. Therefore, repeated post-dialysis blood samplings should be avoided, being unnecessary and even dangerous. Pre-dialysis measurements in serum give a perfect basis for the estimation of post-dialysis blood levels.

Considering the rapid decrease of univariate prediction success, biweekly measurements on RPF are recommended for creatinine, urea, sodium, potassium. Serum calcium and phosphate levels can also be determined from RPF according to need.

Routine control blood tests are required only once per two months, giving a good basis (in combination with known actual dialysate levels) for effective multivariate statistical estimation of the serum levels.

Summarizing the results, we suggest that in patients subjected to chronic intermittent peritoneal dialysis, measurements in serum and dialysate should be combined. The benefits and accuracy of the statistical prediction of laboratory results in routine control of IPD calls attention to similar possiblities in case of any fixed dialysis strategy with calculable short and long-term effects.

## REFERENCES

1. Afifi, A. A., Azen, S. P.: Statistical Analysis: a Computer Oriented Approach. Academic Press, New York 1978, p. 442.
2. Furnival, G. M., Wilson, R. W.: Regression by leaps and bounds. Technometrics **16,** 499–511 (1974)
3. Hildebrand, D. K., Laing, J. D., Rosenthal, H.: Prediction Analysis of Cross Classifications. John Wiley and Sons, New York 1977, p. 311.
4. Lambrey, G., Coevoet, B., de Fremont, I. F., Hardin, I. M., Fournier, A.: Value of biochemical measurements on the residual peritoneal fluid in patients on chronic intermittent peritoneal dialysis. Proc. EDTA **16,** 737–738 (1979)
5. Wichmann, H. E., Vleho, M., Peitz, R., Sieberth, H. G.: Mathematische Analyse der Anwendung des Serumkreatinins bei chronischen Nierenerkrankungen. Nieren und Hochdruckkrankheiten **2,** 69–76 (1979)

*Acta Medica Hungarica, 41 (4), pp. 287—295 (1984)*

# PLASMAPHERESIS AND PERITONEAL DIALYSIS IN THE MANAGEMENT OF RAPIDLY PROGRESSIVE GLOMERULONEPHRITIS

J. SÁMIK, L. GOFMAN, J. NAGY, T. BURGER, B. KEREKES

SECOND DEPARTMENT OF MEDICINE, UNIVERSITY MEDICAL SCHOOL, PÉCS AND REGIONAL BLOOD TRANSFUSION SERVICE, PÉCS, HUNGARY
H-7621 PÉCS, SZÉCHENYI TÉR 5.

(Received May 24, 1983)

Six patients with rapidly progressive glomerulonephritis were given plasmapheresis and peritoneal dialysis by manual technique. At the start of treatment the patients were in renal failure and all but two were practically anuric. Renal biopsy revealed glomerular sclerosis in 4 cases. One of the patients in whom segmental focal fibrosis was found, went into remission. In the other three cases extensive hyaline degeneration was present. One of them was placed on a dialysis program, two died with circulatory failure. In two cases no glomerular sclerosis was found, though extensive changes of the tissues were present. One of these patients went into remission, the other died from respiratory failure consequent upon recurrent pneumothorax and gross pulmonary infiltration.

The only chance of success in the management of rapidly progressive glomerulonephritis is offered by the earliest possible diagnosis. In cases of glomerular sclerosis the chances are very poor.

**Keywords:** rapidly progressive glomerulonephritis, plasmapheresis, peritoneal dialysis

## Introduction

Rapidly progressive glomerulonephritis has the following characteristics: 1. Rapid decline in renal functions, 2. Heavy proteinuria, 3. Glomerular crescent formation in more than 50% of the glomeruli. The process may accompany Henoch–Schoenlein purpura, Goodpasture's syndrome thrombotic microangiopathy (HUS), lupus nephritis, but may also occur as an idiopathic disease.

We are reporting here our observations in 6 cases of rapidly progressive glomerulonephritis treated with plasmapheresis and peritoneal dialysis.

## Methods

In lack of any other possibility, plasmapheresis was carried out by the manual technique. 400 ml blood was drawn from the patient into a flask containing 110 ml ACD stabilizer, centrifuged at 1200 r.p.m. for 30', then the plasma was sucked off and the red blood cell mass was reinfused. Continuous volume replacement from the first blood withdrawal onward was

Send offprint requests to J. Sámik, H-7621 Pécs, Széchenyi tér 5, Hungary

provided for by infusion of identical group plasma with 5% albumin. Each treatment comprised five exchanges.

Peritoneal dialysis was performed by the manual technique via a Tenckhoff catheter 2 to 5 times a week, depending on the renal function. For each treatment 10 to 15 l dialysing fluid was used. The solutions 1D, 2DK and 2D (HUMÁN, Budapest) served as dialysing fluid.

The patients received prednisone 100 mg i.v., cyclophosphamidi, 50 mg i.v. or 150 mg orally. During plasmapheresis the anticoagulant was heparin, in other cases acenocoumarol. All patients were subjected to antibiotic and diuretic treatment.

*Case reports*

*Case 1.* G.F., a female aged 41, had been treated for bronchial asthma in 1941. On Aug. 25, 1981, she had been admitted to a regional hospital for high temperatures, weakness and haemoptysis; extensive pulmonary infiltration, proteinuria and haematuria were found and she was started on antibiotics, steroids and heparin, but her renal functions declined rapidly, and she was transferred to our department on Sept. 22. On admission, sharp vesicular respiration, prolonged expiration with wheezing were heard. The left border of the heart was 2 cm outside the medioclavicular line. The liver was felt 2 cm below the right costal arch. Blood pressure was 230/100 mmHg. ESR was 18 mm/hour, haemoglobin 9 g per dl, haematocrit 24%, leukocyte count 14 000/cu mm, CN 40 mmol/l, creatinine 1240 $\mu$mol/l, Na 140 mEq/l, K 5.0 mEq/l, Cl 196 mEq/l, total protein 55 g/l, albumin 30 g/l, cholesterol 6.25 mmol/l, AST 120 U, C3 70 mg/dl, C4 10.5 mg/dl, ANF negative, immunoelectrophoresis: IgG 12.4 g/l, IgA 3.36 g/l, IgM 0.96 g/l. Anti GBM antibody: 1:64 (pos), Se–FDP $>$ 40.0 $\mu$g/ml. Urine: 500 ml/day, spec. grav. 1010, protein: heavy precipitate, Addis count: erythrocytes 54 M, leukocytes 0, casts 200 000. The urine was sterile. The stools were tarry from haemorrhage from duodenal erosions. The chest X-ray revealed soft, confluent opacities involving both sides, particularly the right upper lobe.

Isotope renography showed an adequate rise of activity and a subsequent decline for 30 sec, followed by a slowly rising curve of accumulation type.

Renal biopsy: light microscopy revealed mesangioproliferative glomerulonephritis with diffuse crescent formation. No hyalinized glomeruli were seen. Immune histology showed massive deposits of IgG and C3 of granular type on the basement membranes and in the mesangium. The Bowman-capsules of a few glomeruli contained fibrin deposits.

On the day of admission the patient was placed immediately on peritoneal dialysis owing to repeated convulsions, and 3 days later plasmapheresis was started. Under the effect of this treatment urine output increased, and on Oct. 7 plasmapheresis and peritoneal dialysis were discontinued. On Dec. 9 she was discharged, CN being 13.3 mmol/l and se-creatinine 199 $\mu$mol/l (Fig. 1).

*Case 2.* G.S., a 20 year old male had been hospitalized on Sept. 28 of 1981 for catarrhal symptoms. 2 days after admission haemoptysis, proteinuria and haematuria were noted. Despite antibiotic therapy he developed sepsis with rapidly deteriorating renal function. The temperature had normalized under the effect of steroids, but he became uraemic and was transferred to our department. On physical examination he appeared anaemic. At the left side there was a supradiaphragmatic dullness with audible crepitations. The liver was felt 4 cm below the costal arch. The legs were oedematous. Blood pressure was 140/80 mmHg.

Laboratory findings: BSR was 90 mm/hour, haemoglobin 8.0 g/dl, haematocrit 26%, leukocyte count 23 000/cu mm, CN 56 mmol/l, creatinine 995 $\mu$mol/l, Na 135 mEq/l, K 7.2 mEq/l, Cl 95 mEq/l, total protein 50 g/l, albumin 25 g/l, cholesterol 6.48 mmol/l, AST 60 U, C3 65 mg/dl ml, C4 55 mg/dl, immune electrophoresis: IgG 12.4 g/l, IgA 1.72 g/l, IgM 1.04 g/l, anti-GBM-antibody negative, CIC negative, se-FDP $>$ 40 $\mu$g/ml. Urine :500 ml/day, specific gravity 1010. Protein: heavy precipitate. Addis count: erythrocytes 278 M, leukocytes 0, casts 100 000. The urine was sterile. Renography: a renogram of isosthenuric type was registered over both kidneys. Light microscopy of the biopsy specimen showed mesangioproliferative glomerulonephritis with diffuse crescent formation. No hyalinised glomeruli were seen. The basement membranes of all glomeruli showed linear IgG staining. In the Bowman spaces sparse fibrin deposits were seen.

In view of the uraemic condition and the high K-level the patient was started immediately on peritoneal dialysis and plasmapheresis. The oedemas disappeared without, however, increasing urine output. By Nov. 6 a pneumothorax developed on the left side, but aspiration was successful. On Dec. 6 he again experienced dyspnoea and had a blood-stained sputum. Chest X-ray showed complete pneumothorax on the left side and an infiltrate involving the

**Table I**

*Clinical data of patients before and after plasmapheresis*

| | Histologic finding | Before treatment | | | | After treatment | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | CN mmol/l | Creat μmol/l | Urine ml/day | Number of PPh | CN mmol/l | Creat μmol/l | Urine ml/day | |
| 1. G.F. f, 42 | Mesangioproliferative glomerulonephritis with diffuse crescent formation. Glomerular hyaline degeneration absent | 40.0 | 1240 | <500 | 8 | 13.3 | 199 | >2000 | Remission |
| 2. G.S. m, 20 | Mesangioproliferative glomerulonephritis with diffuse crescent formation. Glomerular hyaline degeneration absent | 56.0 | 995 | 500 | 16 | 28.9 | 752 | 500 | Death with cardiac failure |
| 3. K.P. f, 54 | Mesangioproliferative glomerulonephritis with focal fibrosis. 80% crescents | 23.7 | 305 | 700 | 4 | 16.3 | 120 | 2000 | Remission |
| 4. P.G. m, 40 | Mesangioproliferative glomerulonephritis. 40% of glomeruli hyalinized, 60% with crescents | 50.8 | 1615 | 1500 | 6 | 29.0 | 995 | 1000 | Haemodialysis |
| 5. Sz.Z. f, 28 | Membranoproliferative glomerulonephritis with diffuse scarring crescents | 28.8 | 548 | 800 | 9 | 39.9 | 820 | <500 | Death. Pulmonary oedema |
| 6. Sz.A. m, 31 | Wegener's granulomatosis | 25.2 | 420 | 1500 | 6 | 16.6 | 323 | 2000 | Death. Sepsis, circulatory failure |

entire right lung. Another chest aspiration was attempted but the patient died with respiratory failure resulting from excessive loss of respiratory surface. Necropsy revealed a haemorrhagic infiltration in the lung. The light microscopic and immunohistologic features of the kidney were identical with those of the biopsy specimen. There were no hyalinised glomeruli. The basement membranes of the lung and choroid plexus showed linear IgG staining (Fig. 2).

*Case 3.* K.P., a female aged 54, had been aware of high blood pressure for the last 5 years. She had been referred for swelling of eyelids and legs to hospital where protein, blood and casts were found in the urine. She was referred to our department for closer investigation and treatment on Oct. 14, 1982. Apart from a moderately enlarged heart, no abnormality of diagnostic relevance was found. Initial oedemas had disappeared. Blood pressure was 170/100 mmHg.

Laboratory findings: ESR was 135 mm/hour, the blood counts were normal, CN was 8.5 mmol/l, creatinine 164 μmol/l, the se-electrolytes were normal. Total protein 54 g/l, albumin 27 g/l, cholesterol 5.35 mmol/l, AST 170 U, $CH_{50}$ 1.42 U, C3 167 mg/dl, immune electrophoresis: IgG 8.4 g/l, IgA 2.24 g/l, IgM 1.2 g/l. Urine: 800 ml/day, spec. gravity 1009, protein: heavy precipitate. Addis count: erythrocytes 160 M, leukocytes 0, casts 0. Bacteriological examination was negative.

Isotope renography: Hippuran uptake of both kidneys was reduced by 50%. Delayed cumulation was followed by slow excretion.

Light microscopy of the biopsy specimen showed mesangioproliferative glomerulonephritis with crescent formation in 80% of glomeruli and focal fibrosis of glomerular segments. Immunhistology revealed deposits of IgM, IgA and C3, in the first place in the peripheral glomeruli, in microgranular form. There were abundant intraglomerular and interstitial fibrin deposits.

Drug treatment failed to halt the rapid decline of renal functions. (CN 23.7 mmol/l, creatinine 305 μmol/l.) Plasmapheresis was started. The fourth exchange was followed by an increase in urine output and an improvement of biochemistry, therefore dialysis was dispensed with. The patient was discharged on December 14; then CN was 16.3 mmol/l, creatinine 120 μmol/l, daily urine output 2000 ml (Table I).

*Case 4.* P.G., a 40 year old male, had known to be hypertensive for 1.5 year. He had been admitted for azotaemia to a provincial hospital where surgical biopsy was done. This revealed mesangioproliferative nephritis with complete destruction of 50% of the glomerul

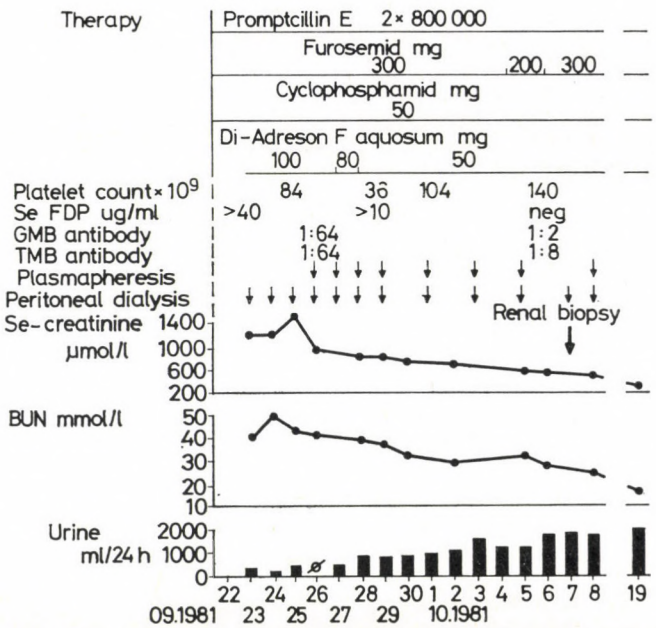



*Fig. 1.* G.F. female aged 41. Diagnosis: Mesangioproliferative glomerulonephritis with diffuse crescent formation

and crescent formation in 60%. Transferred to our department he was found to have a slightly enlarged liver and heart.

Blood pressure was 180/100 mmHg. Laboratory findings: ESR 45 mm/hour, haemoglobin 10.8 g per dl, haematocrit 35%, CN 50.8 mmol/l, creatinine 1615 μmol/l, seelectrolytes normal, total protein 60.0 g/l, albumin 38.0 g/l, cholesterol 3.65 mmol/l, AST 120 U, CRP 35 IU, immune electrophoresis: IgG 11.0 g/l, IgA 2.5 g/l IgM 1.5 g/l. Urine: 1500 ml/day, psec. gravity 1010, protein: heavy precipitate, Addis-count: erythrocytes 456 M, leukocytes 0, casts 0, bacteriology: sterile.

Though his general condition improved under the effect of therapy, the decline of urine output made dialysis necessary. He was placed on the dialysis program as a candidate for renal transplantation.

*Case 5.* Sz.Z., a female aged 28, had experienced swelling of face and legs, in February 1982. Proteinuria, haematuria and high blood pressure had been found. Though the oedema disappeared under a regimen of diuretics, steroids and antibiotics, she developed azotaemia within two months and was transferred to our department on June 14. Physical examination revealed generalized oedema and a dullness of 8 cm above the right diaphragm. On percussion the left border of the heart was found 1.5 cm outside the medioclavicular line. A systolic murmur was heard over all points and there was tachycardia. Percussion revealed free abdominal fluid. The liver was felt 5 cm below the costal arch. Blood pressure was 180/120 mmHg.

Laboratory findings: ESR was 60 mm/hour, haemoglobin 8.6 g/dl, haematocrit 23%, leukocytes 4400/cu mm, CN 27.8 mmol/l, creatinine 548 μmol/l, Na 150 mEq/l, K 5.5 mEq/l, Cl 110 mEq/l, cholesterol 7.6 mmol/l, AST 170 U, CRP 90 IU, C3 20.8 mg/dl, C4 traces, total protein 44 g/l, albumin 3.32 g/l. Urine: 800 ml/day, spec. gravity 1009, protein: heavy precipitate, Addis-count: erythrocytes 336 M, leukocytes 0, casts 0, bacteriology: sterile.

Isotope renography: renogram of background type. The biopsy specimen revealed membranoproliferative glomerulonephritis with diffuse scarring crescents. Immunohistology: IgG formed massive, IgM and C3 less abundant, deposits on the glomerular basement membranes and in the mesangium. In 50% of the glomeruli Bowman's spaces contained abundant fibrin deposits.

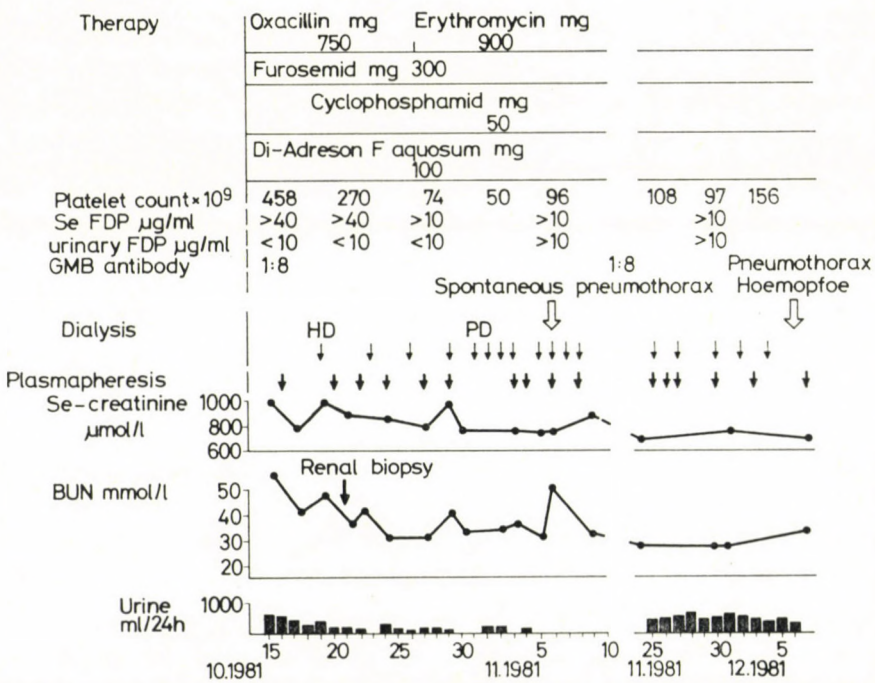



*Fig. 2.* G.S. male, aged 20. Diagnosis: Mesangioproliferative glomerulonephritis with diffuse crescent formation

Treatment failed to renal function. The patient was on dialysis, and he died with circulatory failure on October 5, 1982.

*Case 6.* The disease of Sz.A., a 31 year old male, had started in April 1982 with upper respiratory catarrh, followed by conjunctivitis and haemoptysis. High blood pressure, proteinuria, haematuria had been found. Renal function had declined rapidly and after 4 weeks he had developed uraemia. Renal biopsy had revealed Wegener's granulomatosis. He was admitted to our department on June 14, 1982. On physical examination the liver was felt 6 cm below the costal arch, the spleen was just palpable. Blood pressure was 200/120 mmHg.

Laboratory findings: ESR 33 mm/hour, haemoglobin 16.1 g/dl, haematocrit 42%, CN 17 mmol/l, creatinine 323 μmol/l, se-electrolytes normal, cholesterol 5.23 mmol/l, total protein 60 g/l, albumin 31 g/l, AST 120 U, CRP 100 IU, $CH_{50}$ 1.50 U, C3 126 mg/dl. Urine: 1000 ml/day, spec. gravity 1007, protein: heavy precipitate. Addis count: erythrocytes 150 M, leukocytes 0, casts 0. On bacterial examination the urine was found sterile.

Blood chemistry improved in response to treatment, but on August 12 he became feverish and complained of headache and shortness of breath. X-ray revealed a poorly outlined infiltrate in the right lower lobe and clouded nasal sinuses.

Intensive antibiotic treatment failed to halt the production of sepsis. He died with circulatory failure on August 15.

## Discussion

Evaluation of therapy in glomerulonephritis is made difficult by the spontaneous remissions which may occur occasionally even in its rapidly progressive form [3, 6]. Use of immunosuppressing drugs and steroids seem to fail but combination of such therapy with anticoagulants has been found of benefit [5, 8, 21]. Remissions produced by intensive plasmapheresis were reported by Lockwood [13] and have been confirmed by other authors [11, 12, 14, 16, 18, 19, 20].

The benefits of plasmapheresis may be ascribed to the following factors: 1. It produces a fall in the levels of the circulating immunocomplexes [9, 10], 2. By extraction of the circulating antigens and/or antibodies, it interferes with the formation of local immunocomplexes [9], 3. Far from affecting the clearance function of the mononuclear phagocyte system, it has even been found to stimulate this activity [4], 4. Not only the nephrotoxins are removed but also the levels of the potential nephrotoxins are reduced [2].

Plasmapheresis does not exempt from the use of steroids and immunosuppressors for the depression of immune processes. Cyclophosphamide appears to be the most potent depressant of rebound increase in antibody consequent upon plasmapheresis.

Exchange of 2 to 4 liter plasma per day is generally well tolerated. For volume replacement 5% albumin, fresh or frozen plasma are suitable. Complications are uncommon and generally result from fluctuations of blood pressure due to inadequate volume replacement. A sudden fall in volume may give rise to arrhythmia. Reduction in the immunoglobulin levels is fraught with hazards of infection. Leukocytosis accompanying the inflammatory process results in massive release of proteolytic enzymes and of coagulation activators from the cells, thus adding to the severity of the primary disease.

Protective antibiotic therapy is, therefore, of prime importance. During volume replacement significant amounts of citrate may gain access to the circulation and cause bleedings and/or tetany by binding of $Ca^{++}$. Conversion of citrate to bicarbonate may result in alkalosis during prolonged treatment. The hazard of hepatitis involved by the administration of plasma need not be emphasized.

According to general estimates, plasmapheresis has been found of therapeutic benefit in 40 to 60% of the cases. Gibstein [9] sums up the pertinent observations as follows:

In a material of 31 patients with Goodpasture's syndrome improvement of the renal condition occurred in 19 cases, and recovery from a pulmonary infiltration of major severity in 22 out of 23 cases. This observation is the more remarkable as respiratory failure accounts for the fatal outcome of Goodpasture's syndrome in a high proportion of the cases. 12 out of 16 patients with SLE, 27 out of 40 with crescentic glomerulonephritis, had a remission. The severity of uraemia and anuria, as well as the proportion of glomeruli involved by crescents seem to be inversely related to the success of therapy [9, 17, 22].

Our patients were uraemic at the start of therapy, and all but two were practically anuric. Renal biopsy revealed advanced glomerular sclerosis in 3 cases; all three patients (Nos 4, 5, 6) failed to respond to treatment. Focal sclerosis of glomerular segments was found in one case (patient 3). This patient went into remission. In patients 1 and 2 none of the glomeruli were hyalinised. The first of these patients was discharged in a satisfactory condition and no deterioration of renal function was demonstrable one year later. The second patient died in consequence of recurrent pneumothorax and pulmonary infiltration on the opposite side. At necropsy no glomerular hyalinisation was demonstrable in this case either. Had it not been for the extremely grave complications, this patient too might have possibly survived.

The clinical features of Cases 1 and 2 are consistent with those of Goodpasture's syndrome. In Case 1 anti-GBM-antibody was demonstrable. Renal biopsy was done after intensive therapy, therefore the presence of granular immune deposits is by no means incompatible with the syndrome [1, 16]. Patient 2 revealed linear immune deposits in the kidney, lung and choroid plexus. No anti-GBM-antibody was detectable in this case. This may have the following causes: 1. After onset of the disease the anti-GBM-antibody may have remained in the circulation for a short time only [9]. 2. The antibody of high affinity is rapidly bound to GBM [15], and this may account for the high anti-GBM-antibody levels in less severe forms of Goodpasture's syndrome [3]. 3. The patient had been on high steroid doses before admission to this department.

The prime importance of early diagnosis in the interest of successful therapy clearly emerges from all published observations including the small

number of cases forming the present material. Administration of steroids, immunosuppressors and anticoagulants has to be started without delay. Deterioration of renal function instead of improvement within 14 to 21 days makes plasmapheresis justified. If the patient is uraemic at the time of diagnosis, he should be started immediately on plasmapheresis. An improvement may generally be expected within 4 weeks, but its occurrence at some later time is not beyond the possibilities.

Plasmapheresis and also immunoabsorption [7] which is still in the experimental stage, are expected to improve the chances of remission in rapidly progressive glomerulonephritis.

## Acknowledgement

We are indebted to Dr. G. Deák for studies of the biopsy materials and to Dr. A. Karátson for implantation of Tenckhoff-catheters.

## REFERENCES

1. Agoda, L. C. Y., Stricker, G. E., George, C. R. P.: The appearance of non-linear deposits of immunoglobulins in Goodpasture's syndrome. Am. J. Med. **61,** 407–413 (1976)
2. d'Apice, A. J. F., Kincaid-Smith, P.: Progress in glomerulonephritis. John Wiley, New York 1979, p. 371.
3. Bailey, R. R., Simpson, I. J., Lynn, K. L., Neal, T. J., Doak, P. B., McGiven, A. R.: Goodpasture's syndrome with normal renal function. Clin. Nephrol. **15,** 211–215 (1981)
4. Balow, J. E.: Plasmapheresis therapy. Contemp. Dialysis. March. 9–11 (1981)
5. Berkessy, S., Róna, Gy., Mink, K., Tóth, L., Nagy, K.: A Goodpasture syndromáról. Orv. Hetil. **113,** 1289–1292 (1972)
6. Boros, Gy.: Goodpasture syndroma. Orvosképzés. **48,** 358–368 (1973)
7. Burgstaler, E. A., Pineda, A. A.: Immunoabsorption in an extracorporeal plasma perfusion system: in vitro studies. Artef. Organs **5,** 259–263 (1981)
8. Cameron, J. S., Gill, D., Turner, D. R., Chantler, C., Ogg, C. S., Vosnides, G., Williams, D. G.: Combined immunosuppression and anticoagulation in rapidly progressive glomerulonephritis. Lancet **2,** 923–925 (1975)
9. Gipstein, R. M.: Plasmapheresis, a possible adjunct to treatment of catastrophic crescent nephritis. Contemp. Dialysis. Sept. 33–41 (1981)
10. Hollán, Zs.: Az immuncomplexek klinikai jelentősége és terápiás plasmaferesis hatása. Orvosképzés **53,** 351–362 (1978)
11. Jones, J. V., Bucknall, R. C., Cumming, R. H.: Plasmapheresis in the management of acute systemic lupus erythematosus. Lancet **1,** 709–711 (1976)
12. Lang, C. H., Brown, D. C., Staley, N.: Goodpasture's syndrome treated with immunosuppression and plasma exchange. Arch. Intern. Med. **137,** 1076–1078 (1977)
13. Lockwood, C. M., Boulton-Jones, J. M., Lowenthal, R. M., Simpson, I. J., Peters, D. K., Wilson, C. B.: Recovery from Goodpasture's syndrome after immunosuppressive treatment and plasmapheresis. Br. Med. J. **2,** 252–254 (1975)
14. Lőcsei, L., Kakuk, Gy., Wórum, F., Szabó, J., Kovács, P., Gyarmati, J.: Goodpasture's syndrome: Haemodialysis, pacing, and extrapulmonary oxygenation. Acta Med. Acad. Sci. Hung. **37,** 289–298 (1980)
15. Lyberg, T., Prydz, H., Bakien, K., Hyeraal, H. M.: Effect of immune complex — containing sera from patients with rheumatic disease on thromboplastin activity of monocytes. Thromb. Res. **25,** 193–202 (1982)
16. Melendes, E. E., Forbes, R. D. C., Hollomby, D. J., Katz, M. G.: Goodpasture's syndrome treated with plasmapheresis. Arch. Intern. Med. **140,** 542–543 (1980)

17. Neilson, E. G., Phillips, S. M., Agus, Z.: Plasmapheresis in culminating glomerulonephritis. Relapse after prolonged remission. Arch. Intern. Med. **141,** 852–854 (1981)
18. Rossen, R. D., Duffy, J., McCredie, K. B.: Treatment of Goodpasture's syndrome with cyclophosphamide, prednisone and plasma exchange transfusion. Clin. Exp. Immunol. **24,** 218–222 (1976)
19. Russ, G. R., d'Apice, A. J. F.: Plasma exchange and immunosuppression in crescentic glomerulonephritis. Proc. 8th Int. Congr. Nephrology, Athens 1981, pp. 667–673.
20. Schmidt, E., Falkenhagen, D., Preussner, D., Ressenow Holtz, M., Klinkmann, H.: Plasma separation and plasmapheresis — a comparative study. Plasma Exchange. F. K. Schattauer Verlag, Stuttgart–New York 1980, pp. 99–103.
21. Suc, J. M., Durand, D., Conte, J., Conte, M. M., Orfila, C., Ton That, H., Duchet, J. P.: The use of heparin in the treatment of idiopathic rapidly progressive glomerulonephritis. Clin. Nephrol. **5,** 9–13 (1976)
22. Varren, S. E., Mitas, J. A., Golbus, S. M.: Recovery from rapidly progressive glomerulonephritis. Arch. Intern. Med. **180,** 141–175 (1981)

*Acta Medica Hungarica, 41 (4), pp. 297—302 (1984)*

*Immunology*

# NORMALIZATION OF ELECTROPHORETIC MOBILITY OF LYMPHOCYTES IN PATIENTS WITH PRIMARY IMMUNOGLOBULIN DEFICIENCY TREATED WITH INTRAVENOUS IMMUNOGLOBULINS

C. Ricci, E. Minetto, G. Cascio, A. De Stefano, M. Basso, P. Borelli, D. Bozzone, D. Delia, R. Baldassi

MEDICAL DEPARTMENT UNIVERSITY HOSPITAL
TORINO-10129, CORSO POLONIA 14, ITALY

(Received July 19, 1983)

The behaviour of lymphocyte sub-populations and lymphocyte electrophoretic mobility has been studied in seven patients with agamma/hypogammaglobulinaemia before and after gammaglobulin therapy.

**Keywords:** L.E.M.: Lymphocyte electrophoretic mobility, gamma-globulin therapy, primary immunodeficiency, X-linked agammaglobulinaemia, monoclonal antibodies.

**Abbreviations:**

X–L.A: X-linked agammaglobulinaemia
CVH: Common variable hypogammaglobulinaemia
SRFC: Sheep-rosette forming cells
PBL: Peripheral blood lymphocytes
MoAb: Monoclonal antibody
SIgI: Serum immunoglobulin infusion (Sandoz)
SIg: Surface immunoglobulins
L.E.M.: Electrophoretic mobility of lymphocytes
P.W.M.: Pokeweed mitogen

## Introduction

It has been reported that the humoral immune response in experimental systems may be reduced by immunoglobulins. In addition, an excess suppressor activity has been described in patients with hypogammaglobulinaemia receiving gammaglobulin therapy.

Waldmann was the first to observe that suppressor cells may play an important role in the pathogenesis of common variable hypogammaglobulinaemia (CVH). Therefore we have studied the in vivo cellular effect of gammaglobulins (Sandoglobulin® Sandoz) injected intravenously in seven patients with primary immunodeficiency. The aims of this preliminary study were to study

Send offprint requests to: Ciro Ricci, M.D., Via Piazzi 41, Torino, 10129, Italy

**Table I**

*Clinical and immunological data of seven patients with*

| Name | Diagnosis | Age (years) | Sex | Pre-infusion | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | W.B.C. ($mm^3$) | PBMC percent | S.Ig (percent) | | | |
| | | | | | | IgG | IgA | IgM | IgD |
| S. Rb. | X—L. A. | 34 | M | 2000 | 27 | 0 | 2 | 3 | 6 |
| S. Rs. | X—L. A. | 34 | M | 5600 | 42 | 0 | 2 | 3 | 5 |
| B. M. | X—L. A. | 16 | M | 7000 | 45 | 0 | 0 | 0 | 0 |
| S. C. | C.V.H. | 57 | F | 8200 | 41 | 1 | 2 | 3 | 6 |
| R. S. | C.V.H. | 38 | F | 5800 | 50 | 1 | 1 | 2 | 4 |
| B. A. | C.V.H. | 48 | F | 4600 | 30 | 0 | 1 | 2 | 4 |
| A. E. | C.V.H. | 31 | M | 21000 | 32 | 0 | 4 | 6 | 8 |

**Table II**

*Surface markers of PBMC and percentage of positive PBMC*

| Name | Diagnosis | Age (years) | Sex | Pre-infusion | | | |
|---|---|---|---|---|---|---|---|
| | | | | W.B.C. ($mm^3$) | PBMC (percent) | SRFC (percent) | |
| | | | | | | A | T |
| S. Rb. | X-L. A. | 34 | M | 2000 | 27 | 47 | 69 |
| S. Rs. | X-L. A. | 34 | M | 5600 | 42 | 54 | 67 |
| B. M. | X-L. A. | 16 | M | 7000 | 45 | 64 | 77 |
| S. C. | C.V.H. | 57 | F | 8200 | 41 | 64 | 77 |
| R. S. | C.V.H. | 38 | F | 5800 | 50 | 60 | 72 |
| B. A. | C.V.H. | 48 | F | 4600 | 30 | 55 | 76 |
| A. E. | C.V.H. | 31 | M | 21000 | 32 | 43 | 68 |

the functional property of lymphocytes before and after immunotherapy; and to check the activation of suppressor activity after gammaglobulin infusion.

## Material

We treated three patients with X–L. agammalobulinaemia (X–L.A) and four with CVH. The criteria for the diagnosis of X–L.A were: male sex, onset in infancy or early childhood, recurrent pyogenic infections, serum immunoglobulins virtually absent, inability to make antibodies after antigenic stimulation, intact cellular immunity, sparse lymphoid tissue without plasma cells, family history of affected males. Those for diagnosis of CVH were: absence of specific antibodies and a failure to mount an antibody response to standard antigen challenge.

## Methods

*Isolation of lymphocytes and surface marker studies*

Peripheral blood lymphocytes were isolated from heparinized blood by Ficoll–Hypaque density gradient. Sheep rosette forming cells (SRFC) and membrane Ig were studied as described previously [6].

*Monoclonal antibody (MoAb) of lymphocytes by indirect immunoflourescence*

Three MoAb described previously by other authors were used. Briefly, OKT3 MoAb binds to all mature T cells, whereas regulatory T-helper and T-suppressor cells are restricted to $OKT4^+$ and $OKT8^+$ restricted cells, respectively. To determine the frequency of po-

*primary Ig Deficiency before and after i.v. Ig therapy*

| Serum Ig (mg/dl) | | | W.B.C. ($mm^3$) | PBMC per cent | Post-infusion S.Ig (per cent) | | | | Serum Ig (ml/dl) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| IgC | IgA | IgM | | | IgG | IgA | IgM | IgD | IgG | IgA | IgM |
| 89 | 30 | 29 | 6200 | 26 | 0 | 0 | 1 | 6 | 410 | 10 | 34 |
| 24 | 2 | 38 | 7300 | 41 | 0 | 1 | 6 | 4 | 470 | 11 | 34 |
| 197 | 0 | 0 | 10300 | 49 | 0 | 0 | 0 | 0 | 218 | 0 | 0 |
| 133 | 21 | 7 | 3200 | 49 | 0 | 2 | 5 | 4 | 265 | 42 | 17 |
| 10 | 8 | 15 | 7400 | 54 | 0 | 0 | 1 | 10 | 365 | 0 | 0 |
| 100 | 0 | 0 | 5600 | 32 | 0 | 2 | 3 | 5 | 415 | 0 | 0 |
| 36 | 0 | 0 | 21700 | 30 | 0 | 3 | 5 | 6 | 174 | 17 | 7 |

*studied with a panel of MoAb before and after i.v. Ig infusion*

| OK (percent) | | | Ratio $T_4/T_8$ | W.B.C. ($mm^3$) | PBMC per cent | Post-infusion SRFC (per cent) | | OK (percent) | | | Ratio $T_4/T_8$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| $T_3$ | $T_4$ | $T_8$ | | | | A | T | $T_3$ | $T_4$ | $T_8$ | |
| 100 | 57 | 42 | 1.36 | 6200 | 26 | 44 | 69 | 90 | 28 | 42 | 0.67 |
| 100 | 56 | 43 | 1.30 | 7300 | 41 | 44 | 74 | 90 | 36 | 45 | 0.80 |
| 100 | 50 | 39 | 1.28 | 10300 | 49 | 42 | 60 | 80 | 48 | 30 | 1.60 |
| 100 | 50 | 53 | 0.94 | 3200 | 49 | 71 | 76 | 100 | 33 | 28 | 1.18 |
| 100 | 45 | 46 | 0.98 | 7400 | 54 | 67 | 71 | 90 | 24 | 48 | 0.50 |
| 90 | 41 | 46 | 0.89 | 5600 | 32 | 55 | 82 | 100 | 37 | 36 | 1.02 |
| 100 | 50 | 47 | 1.06 | 21700 | 30 | 53 | 80 | 98 | 20 | 50 | 0.40 |

sitive cells, peripheral blood lymphocytes (PBL) were incubated with appropriate amounts of each MoAb for 30 min at 4 °C in complete medium with 10% fetal calf serum, washed, counter-labelled with fluorescent goat anti-mouse IgG, washed again, and then examined with a Leitz ultraviolet microscope equipped with vertical illumination (°). Supernatant from the P3×P3–Ag8 myeloma cell line was used as a negative control for background staining of each cell population, as suggested by Ortho Pharmaceuticals for visualization of positive cells.

These monospecific reagents were used for indirect immunofluorescence microscopy.

*Determination of electrophoretic mobility of lymphocytes (L.E.M.)*

The following equipment was used: direct current source, incorporated microscope with a ×25 eye-piece provided with a calibrated graticule and a modified Zeilers cytopherometer. The lymphocytes were placed in the cytopherometer chamber, the 3.5 mA, 60 V circuit was closed, and the time taken by the edge of a cell to cover two full squares (100 $\mu$) was measured.

Then current was reversed and the time taken by the cell to come back over the same distance was measured. The test was performed with 200 cells.

## Results

All patients displayed clinical improvement (lack of infections, no absence from work) after a standard infusion of 100 mg/kg/month of Sandoglobulin® (SIgI).

(°) Gift of Edoardo Agnelli Foundation, Torino

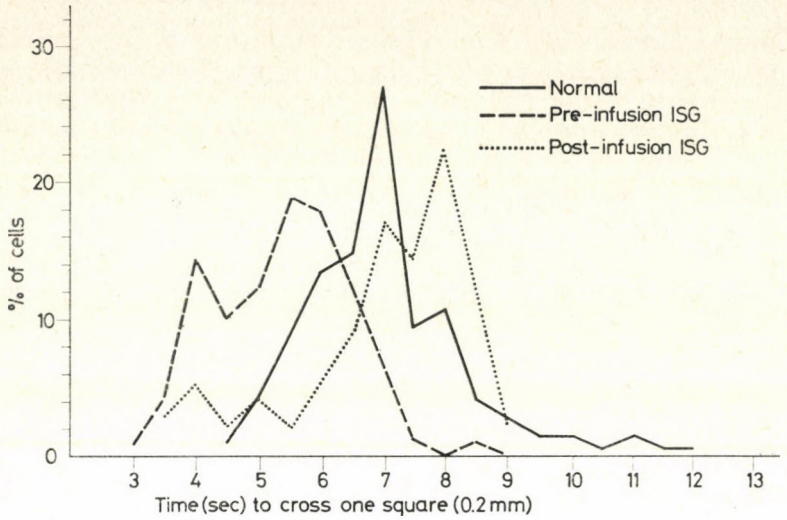



*Fig. 1.* Modification of the electrophoretic mobility of lymphocytes induced by IgG therapy in patients with primary immunoglobulin deficiencies

As can be seen in Table I, Ig always rose for 30 days, but after SIgG lymphocyte surface Ig (SIg) was not modified significantly. In particular, there was no significant depression of SIgM and SIgD markers.

Table II shows that total and active SRFC were not altered after immunotherapy. The T-helper subpopulation decreased significantly in all patients, whereas the T-suppressor sub-population was reduced in 3/7 patients only (B.M., S.C. and B.Al), so that their T4/T8 ratio was positive.

Lastly, as shown in Fig. 1 and Table III, L.E.M. improved significantly in 6/7 patients.

**Table III**

*Modifications of electrophoretic mobility of lymphocytes before and after gammaglobulin therapy in patients with primary immunoglobulin deficiencies*

| Name | Diagnosis | Age (years) | Sex | Pre-infusion | | | Post-infusion | | | Variation |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | W.B.C. ($mm^3$) | PBMC per cent | E.M.L. (sec) | W.B.C. ($mm^3$) | PBMC per cent | E.M.L. (sec) | E.M.L. per cent |
| S.Rb. | X-L. A. | 34 | M | 2000 | 27 | 5.86 | 6200 | 26 | 7.95 | +36 |
| S.Rs. | X-L. A. | 34 | M | 5600 | 42 | 4.12 | 7300 | 41 | 6.67 | +62 |
| B.M. | X-L. A. | 16 | M | 7000 | 45 | 6.48 | 10300 | 49 | 7.91 | +22 |
| S.C. | C.V.H. | 57 | F | 8200 | 41 | 6.02 | 3200 | 49 | 8.10 | +35 |
| R.S. | C.V.H. | 38 | F | 5800 | 50 | 5.24 | 7400 | 54 | 6.91 | +32 |
| B.A. | C.V.H. | 48 | F | 4600 | 30 | 5.12 | 5600 | 32 | 5.00 | − 2 |
| A.E. | C.V.H. | 31 | M | 21000 | 32 | 4.06 | 21700 | 30 | 6.88 | +69 |

## Discussion

Our in vivo data failed to confirm the experimental results of Durandy et al. [2]. There was no decrease in the SIg of PBL, i.e. Ig synthesis was apparently not inhibited. (These results, however, should be checked by in vitro stimulation of B-lymphocyte activity by P.W.M.). The decrease in the T-helper subpopulation after SIgI may be due to a feedback mechanism with depression of T-helper activity and transient changes in T-suppressor activity. Lastly, the normalization of L.E.M. should be emphasized. This result is difficult to explain. It is possible that SIgI may block the Fc receptors of lymphocytes, thus slowing down their electrophoretic mobility.

Unlike rosette assays, which are influenced by factors such as the SRBC source, cell electrophoretic mobility is determined by direct physical measurement. It is a useful immunological parameter, particularly in the delineation of PBL subpopulations.

Our L.E.M. studies were always accompanied by a parallel study using other cell markers, such as SIg SRFC and complement receptors, etc.

A marked variability in classic cell surface markers with regard to the function and surface profiles of lymphocyte subpopulations was indeed noted in primary humoral immunodeficiencies, and a good correlation was found between some of these markers and the L.E.M. profiles.

In conclusion, SIgI appears to normalize the lymphocyte membrane of patients with primary immunodeficiencies.

## REFERENCES

1. Ammann, A. J., Ashman, R. F., Buckley, R. H., Hardie, W. R., Krawtmann, H. J., Nelson, J., Ochs, H., Stiehm, E. R., Tiller, T., Wara, D. W., Wedgwood, R.: Use of intravenous $\gamma$-globulin in antibody immunodeficiency. Results of a multicenter controlled trial. Clin. Immunol. Immunopath. **22,** 60–68 (1982)
2. Durandy, A., Fische, A., Griscelli, C.: Induction by gamma-globulin therapy of T and B lymphocyte dysfunction in the in vitro PWM system. In: Primary Immunodeficiencies. Eds M. Seligmann, W. H. Hitzig. 16th Proc. Inserm Symposium N. 16, Elsevier—North-Holland Biomedical Press, Amsterdam-New York 1980.
3. Hitzig, W. H., Joller, P. W., Pluess, H. J., and Seger, R.: Treatment of Immunodeficiency syndromes in primary immunodeficiencies Proc. 16th Inserm Symposium. Eds M. Seligmann, W. H. Hitzig Elsevier—North-Holland Biomedical Press, Amsterdam-New York 1980.
4. Kaplan, J. M., Uzgiris, E. E., Logwood, S. H., Cunningham, T. J., Steiner, D.: Characterization of Lymphocytes in normal individuals and cancer patients by electrophoretic mobility and rosette formation with sheep erythrocytes: is mobility a reliable parameter? In: Cell Electrophoresis: Clinical Application and Methodology. Eds Preece, A. W., Sabolovic, D., Proc 11th Inserm Symposium North-Holland Publishing Company, Amsterdam New York 1979.
5. Pandolfi, F., Quinti, I., De Rossi, G., Frielingsdorf, A., Tonietti, G., Paqualetti, D., Aiuti, F.: Abnormalities of regulatory T-cell sub-populations in patients with primary immunoglobulin deficiencies. Clin. Immunol. Immunopath. **22,** 323–331 (1982)
6. Ricci, C., Cascio, G., Infelise, V. E., Borelli, P., Borio, T., Bozzone, D., Uberti, M., Vitrano, G., Carbone, G.: Coexpression of multiple immunoglobulin heavy chain classes on human leukemic B lymphocytes. Panminerva med. **23,** 133–139 (1981)

7. Sabolovic, D., Sabolovic, N.: Clinical applications of cell electrophoresis. In: Cell electrophoresis, clinical application and methodology. Eds Preece A. W., Sabolovic, D., Proc. 11th Inserm Symposium North-Holland Publishing Company, Amsterdam, 1979.
8. Schurman, R. K. B., Gelfand, E. W., Touraine, J. L., Van Roos, J. J.: Lymphocyte membrane abnormalities associated with primary immunodeficiency disease. In: Primary Immunodeficiences, eds M. Seligmann, W. H. Hitzig, Proc. 16th Inserm Symposium, Elsevier Amsterdam, — New York 1980.
9. Tuy, F. P. D., Bach, M. A., Durand, Y. A., Griscelli, C.: T cell subsets analyzed by monoclonal antibodies. In: Primary immunodeficiencies, Proc. 16th Inserm Symposium, eds M. Seligmann, W. H. Hitzig, Elsevier, Amsterdam–New York, 1980.
10. Waldmann, T. A., Broder, S., Blaese, R. M., Durm, M., Blackman, M., Strober, W.: Role of suppressor T-cells in the pathogenesis of common variable hypogammaglobulinaemia. Lancet **2**, 609–613 (1979)
11. Waldmann, T. A., Broder, S., Goldman, C. K., Marshall, S., Muul, L.: Suppressor cells in common variable immunodeficiency in Primary Immunodeficiencies. Proc. 16th Inserm Symposium. Eds. Seligmann, S., W. H. Hitzig. Elsevier North-Holland Biomedical Press, Amsterdam, — New York 1980.

*Acta Medica Hungarica, 41 (4), pp. 303—318 (1984)*

# *Book reviews*

*Cancer control in the countries of the Council of Mutual Economic Assistance.* Edited by N. P. NAPALKOV and S. ECKHARDT. Publishing House of the Hungarian Academy of Sciences. Budapest 1982. Price: US $ 35.—

This is the English version of a book which was first published in Russian in 1980. It is a work of special value, being one of the valuable results of a collaboration maintained since many years between the Comecon countries, in the frame of the project: "Complex research on malignant neoplasms". In the publication the leading oncologists of the Comecon countries give detailed information — each in their own country — about the incidence of tumourous diseases, the structure and system of oncological nstitutions where cancer patients are treated. Each chapter begins with a brief summary on ithe geographic, economic and demographic characteristics of the country, giving surveys on the past of the anti-cancer fight. Information is given about the system of registration of cancer patients, the data of cancer screening for the early discovery of certain types of tumour. One can read about the institution where therapeutic treatment is given to cancer patients, the system of education and postgraduate training of oncological specialists, physicians and paramedical personnel, as well as about the health education of the public on the problems of cancer. The last 3 chapters are of special significance, in which the editors give a review on the further programme of oncological coordination, and the prospects of coordination of the fight against cancer in the Comecon countries. The book including 109 tables and more than 40 diagrams provides many useful data regarding cancer morbidity, mortality and the results of the anti-cancer fight in the Comecon countries.

This English version will in all probability promote that the many useful data and experience contained in the book become international public property.

K. LAPIS

H. STALSBERG (ed.): *An International Survey of Distributions of Histologic Types of Tumours of the Testis and Ovary.* UICC (International Union Against Cancer). Geneva 1983. Publisher: H. Huber, Berne. Price: US $ 24.—

This volume, based on the proceedings of the conference held in Ibadan (Nigeria) in March 1981, on the gonadal tumours, contains 36 papers supplied with diagrams. The book falls into four main sections. The first deals with the tumours of the testis, the second with those of the ovary, in accordance with the cancer register and classification evaluated at previous UICC meetings. In the third the patient material of hospitals in Africa, Japan,

Great-Britain, Norway and America are discussed on a similar basis. In the fourth we find a comparative statistical analysis by H. Stalsberg of the ovarian and testicular tumours of various types. The meeting was attended by numerous scientists of wide repute, including W. Johnson (New Orleans), E.E. Scully (Boston), R.G.F. Parker and J.A. Waterhouse (Great-Britain), A.O. Williams (Nigeria) and T. Sund, who performed the automated analyses.

As regards the tumours of the testis, there has been general agreement on their prevalence in the age groups 20–60, but they have been found less prevalent in the coloured than in the white populations. Their incidence is, however, unrelated to geographic factors. It is of interest to note that for instance in Peru these tumours have a high incidence in children, and that seminoma is the predominant type. According to a New Orleans study-group, hormonal imbalance during pregnancy is an essential aetiological factor of tumours of this type and accounts in all probability for their high incidence in cryptorchidism. Its incidence in Japan is 0.97 in 100 000. In Birmingham it is on the increase and attains 2.69 in 100 000.

Classification problems of ovarian tumours are dealt with in a separate chapter by Scully. Details of 175 tumours received for typing from 12 pathologists are given. Only 62% of the consultants agreed on the type of tumour in question. This is attributed by the author to the inaccuracy of some criteria. It is in the first place the classification of undifferentiated carcinoma and of granulosa-cell tumours which gave cause to errors. It has been confirmed that the incidence of ovarian tumours is on the increase. In the USA it belongs to the common causes of death, particularly in the white population. The clear-cell carcinoma is the most benign form, followed by endometrial and mucinous tumours, which are still of better prognosis than the serous and undifferentiated forms. The incidence of ovarian carcinoma in the various countries all over the world is listed in detail.

From section 3, which compares the figures for inpatients with those for outpatients, it emerges that young patients are the majority of inpatients. This is attributed to socio-economic factors and to the type of tumour. The data coming from the various centres are too inhomogeneous to be summed up. A comparative statistical analysis of the testicular and ovarian tumours is given on a few pages at the end of the book. Close relationships have been found between ovarian and testicular tumours of every type. For instance, seminoma of the testis and dysgerminoma of the ovary are of the same incidence.

The book provides valuable information to all those interested in tumours of the testis and ovary, especially to pathologists, oncologists, gynaecologists and urologists.

Magdolna GAÁL

*TEM Classification of Malignant Tumours.* A brochure of checklists. UICC Technical Report Series, Vol. 5. Geneva, 1982

The Union International Centre le Cancer (UICC) is an international union of 182 cancer research centres and oncological societies. One of its activities is the collection and registration of data related to malignant disease on a standardized basis of uniform criteria. It is in the interest of these tasks that the present brochure of checklists covering all regions of the organization has been issued. Each checklist shows, in addition to a sketch of the region, the data of pretreatment and of postsurgical pathohistological classification. On the backside directions how to fill out the printed lists for each organ are found.

The printed checklists have the advantage of excluding any possible omission of essential data, moreover they provide directions for the registration of the entries on the basis of the international standards approved by UICC, thus being suited for comparison, even for purposes of an international cooperative evaluation. There is one checklist for each region, intended for

reproduction. This is granted to any applicant at request by UICC, with the reservation of copyright (UICC, 3, rue due Conseil-Général, 1205 Genève, Switzerland). Two slender brochures issued by UICC, TNM Classification of Malignant tumours (Geneva, 1982) and TNM Classification of Paediatric Tumours (Geneva, 1982), both edited by MH. Harmer serve as supplements to the checklists by providing detailed directions for the classification of each type of tumour with respect to grades N and M. The topographic definitions are based on the Classification of Diseases for Oncology of WHO (ICD–O). It is noted that the Morphological field of ICK–O corresponds to the respective classification of SNOMED.

B. SZENDE

UICC Technical Report Series, Volume 74. *Hepatocellular Carcinoma.* A Series of Workshops on the Biology of Human Cancer. Report No 17. Edited by Kunio Okuda and Ian Mackay. Union Internationale Contre le Cancer. Geneva, 1982

This book originates from a conference held at the UICC headquarters in Geneva, on November 22–26, 1980, with the purpose of reviewing the major problems of hepatocellular carcinome (HCC). The participants, acknowledged experts on various aspects of hepatocarcinogenesis were invited by Dr. Donald Metcalf, chairman of the UICC Programme on Experimental Oncology, to discuss "what is known and what is unknown, and to present opinions on what can be done and what should be done", as Dr. Okuda put it in the preface.

The book fulfils these expectations. It consists of ten chapters written by one or, mostly, by a group, of the participants. The chapters are based on working papers which had been prepared by the same authors and then discussed and critically analysed by all of the workshop members. Thus the final version of the chapters has become the responsibility of the entire group.

I. S. Mackay's interesting and thoughtful introduction (Chapter I) is followed by a part in the epidemiology (Chapter II by K. Okuda and P. Bessley) and one on the pathology (Chapter III by N. Nayak) of HCC. Chapter IV (S. Goldfarb and L. L. Slaughter) includes the biology of rodent HCC and their putative precursors. Chapter V (R. P. Beasley, B. Blumberg, H. Popper and S. Wain–Hobson) on hepatitis B virus (HBV) in relation to HCC is the longest and perhaps the most interesting one, including lesions in animals produced by viruses related to HBV, its molecular biology, and the pathogenesis of HCC associated with HBV. The next chapter summarizes the data of chemical hepatocarcinogenesis. They provide suggestive evidence for a causal relationship between aflatoxin ingestion and the incidence of HCC in man, whereas the role of other, potentially hazardous substances in human hepatocarcinogenesis has not yet been proved. Chapter VII (M. C. Kew, M. Newberne and H. Popper) deals with the importance of other aetiological processes in HCC and concludes that alcohol, the cirrhotic process itself, nutritional and genetic factors may play an indirect role by increasing the susceptibility to environmental aetiological agents which are thought to be responsible for HCC. The following two chapters discuss the significance in HCC of tumour markers, especially that of alpha fetoprotein (M. C. Kew and P. M. Newbern) and the various interactions of host and tumour (M. C. Kew, I. R. MacKay and H. Thomas). The aim of the final chapter is to draft guidelines with recommendations for future research.

The chapters are, by and large well written. The readers will find themselves learning many things even from those chapters the content of which they thought they knew and understood well. Any clinician and pathologist interested in hepatology will find some important information in the book.

I. BARTÓK

IARC Monographs on the *Evaluation of the Carcinogenic RISK of Chemicals to Humans.* Vol. 29 (1982), (Price: Sw. fr. 60.—), 30 (1983), (Price: Sw. fr. 60.—) and Suppl. No. 4. (1982), Price: Sw. fr. 60.—)

The IARC Monographs are well recognized as authorative sources of information on the carcinogenicity of different industrial and environmental chemicals. The first survey, made in 1976, indicates that the monographs are consulted routinely by various agencies in 24 countries. Each volume is printed in 4000 copies and distributed by the WHO publication service.

Up to November 1981, 29 volumes of the Monographs were published. A total of 585 compounds or occupational exposure was evaluated. For 44 chemicals, a positive association or suspicion has been found concerning human cancer. For 147 of them, there was sufficient evidence of carcinogenicity in experimental animals. These critical analyses of collected data are intended only to assist national or international authorities in formulating decisions concerning preventive measures, but no recommendations are given as to legislative consequences.

*IARC Monographs No. 27: Some industrial chemicals and dyestuffs*, pp. 416

This volume of IARC Monographs covers 18 of miscellaneous industrial chemicals including such important and widely distributed environmental contaminants like benzidine and three other benzidine-based dyes (Direct Black 38, Direct Blue 6 and Direct Brown 95). These chemicals are carconogenic to animals and also to humans and they are metabolically degraded to form benzidine which is a well-known human carcinogenic chemical.

From these dyestuffs thousands of kgs are produced and used every year to dye cellulose, silk, paper, wood, textiles, hair. Although some of these chemicals had been evaluated previously but as recently new data had become available, a new evaluation had been made in this volume.

*Benzene* was also considered previously by a Working Group in 1974. Since that time some new data have become available which are also incorporated into this monograph. Benzene is a natural constituent of crude oil, therefore occupational exposure to benzene occurs in numerous industries. It is present as a component of many fuels and as an impurity in organic chemicals made of them. There is limited evidence that benzene was carcinogenic in experimental animals. It has been shown that human exposure to benzene or benzene containing mixtures may damage the haemotopoictic system. The relationship between benzene exposure and the development of acut myelogenous leukaemia has been established in epidemiological studies.

The other important compound, *formaldehyde*, was also considered in this volume of IARC Monographs. The Group was aware of experiments in progress at New York University and the Chemical Industries Institute of Toxicology; but since the final results of those studies have not yet been published, they could not be taken into account while evaluating the possible carcinogenicity of formaldehyde. There is sufficient evidence of formaldehyde gas being carcinogenic in rats. The epidemiological studies provide inadequate evidence to assess the carcinogenicity of the compound in man.

Epidemiological assessment of the carcinogenicity of the chemical compounds evaluated in this volume has several features not considered previously in the IARC Monographs. There has been formal mathematical evaluation of the statistical power of the studies considered, or simply a quantitative estimation of the carcinogenic risk. These estimates were based on data from human epidemiological studies and did not include the results of animal studies.

*IARC Monographs No. 30:* Miscellaneous *Pesticides*, pp. 424

Since pesticides, herbicides and fungicides are ubiquitous and many of them have been shown to be oncogenic in animal studies and active in short-term tests, the risk of cancer from exposure to these compounds is of concern. In most parts of the world almost everyone is exposed to pesticides, but in this review only the cancer risk factors of those population who are occupationally exposed to them is discussed. Epidemiological studies of exposure to arsenical pesticides were described and evaluated in a previous monograph (IARC, 1980) and are therefore not included here. Monographs on other pesticides, such as DDT, phenoxyacetic acid herbicides, chlordane/heptachlor, were included in Volumes 5, 7, 12, 15 and 20 of the Monographs series.

The monographs on nine insecticides, five herbicides, three fungicides and the rodenticide 1-naphthylthiourea (ANTU) have been included in this volume.

Several pesticides containing secondary or tertiary amino groups can be nitrosated by a variety of nitrosating agents to form N-nitroso compounds. A possibility also exists that N-nitroso compounds could be formed in vivo from pesticide residues. Under other circumstances, N-nitroso derivatives can be formed during manufacture and storage as we can see in the Appendix of this volume, which includes a table of published studies on the testing of pesticide which may contain N-nitrosocompounds.

Apart from possible carcinogenic risks, some compounds particularly the organophosphates, present severe toxic hazards to pesticide handlers. Signs and symptoms of acute systemic poisoning that are similar in all organophosphate poisonings are related to inhibition of acethylcholinesterase.

Organophosphates are usually absorbed rapidly by inhalation, ingestion and through the skin but do not accumulate in the body. Some organophosphates require metabolic activation in order to act as esterase inhibitors, while others do not. Detoxyfying metabolism in man is usually very rapid and has been shown in these reviews to be very similar to that in laboratory animals. Measurement of such metabolites in urine can be used to monitor pesticide absorption. Six cases of aplastic anaemia, one of which subsequently developed into leukaemia, and one leukaemia have been reported in children after domestic application of organophosphorous pesticides. Several reports have been published about the presence of pesticides in residues, particularly DDT and dieldrin in tissues of subjects with lung, gastrointestinal, and breast cancer and generalized metastatic carcinomas, and in tissues of control subjects. Increased carcinogenic risks of malignant lymphoma of the histiocytic type and soft tissue sarcomas have been demonstrated in case-control studies of people in Sweden occupationally exposed to phenoxyacetic acid and chlorophenol herbicides, presumably contaminated with tetrachlorodibenzo-para-dioxin (TCDD). Case reports of cutaneous non-Hodgkin lymphoma and epidemiological results from three case control studies from one country and from small cohorts in another country provide limited evidence of the carcinogenicity of phenoxy acids and chlorophenols. Other epidemiological studies provide inadequate evidence to evaluate the carcinogenicity of organic pesticides, as a broad class or as individual compounds.

*IARC Monographs Supplement No. 4: Chemicals, Industrial Processes and Industrial Associated with Cancer in Humans:* IARC Monographs, Vol. 1–29, pp. 467

An international "ad hoc" Working Group of 20 experts in cancer research met in Lyon in January 1979, to re-evaluate the epidemiological and carcinogenicity data of 54 chemicals, chemical or industrial processes which had been evaluated in Volumes 1–20 of IARC Monographs. Of these, 18 chemicals, and industrial processes were considered to be

carcinogenic for humans. In the present volume of IARC Monograph Supplement 4 the Working Group concluded that 7 industrial processes and occupational exposure to 23 chemicals and groups of chemicals are causally associated with cancer in humans. This new list of human carcinogens (see Table I) contains only those exposures which were available for re-evaluation on the basis of summarized data on carcinogenecity in humans.

The present state of knowledge does not permit the selection of specific tests which would precisely identify all classes of potential carcinogens, although certain systems are more sensitive to some substances than to others. Results from several studies of the predictive value of various short-term tests show that some chemicals of proven carcinogenicity in experimental animals are, as far as could be judged, inactive in tests that utilize DNA or chromosomal damage as endpoint. These include, for example, certain hormones, metals and tumour promoters. At present no objective criteria exist to interpret data from studies in experimental animals or from short-term tests directly in terms of human risk. Thus, in the *absence of sufficient evidence* from human studies, evaluation of the carcinogenic risk to humans was based on consideration of both the epidemiological and experimental evidence. The breadth of the categories of carcinogenic evidence defined above all the substantial variation within each class.

*Group 1*

This category was used only when there was *sufficient evidence* from epidemiological studies to support a causal association between exposure and cancer.

**Table I**

| Chemicals | | Industrial processes |
|---|---|---|
| 4-aminobiphenyl | asbestos | auramine manufacture |
| analgesic mixture | arsenic | boot and shoe manufacture |
| azathioprine | benzene | furniture manufacture |
| benzidine | chlornaphazine | isopropyl alcohol manufacture |
| bis/chloromethyl/ether | busulfan | nickel refining |
| chemotherapy (MOPP) | chlorambucil | rubber industry |
| chromium and certain | chromium compounds | underground haematite mining |
| conjugated oestrogens | cyclophosphamide | (with exposure to radon) |
| diethylstilboestrol | melphelan | |
| methoxalene + UV (PUVA) | mustard gas | |
| 2-naphthylamine | soots, tars and oils | |
| treosulphane | vinyl chloride | |

*Group 2*

This category includes exposures for which at one extremity, the evidence of human carcinogenicity is almost "sufficient" as well as exposures for which, at the other extremities, it is inadequate. To reflect to this range, the category was divided into higher (Group A) and lower (Group B) degrees of evidence.

*Group 3*

The chemicals, industrial processes or occupational exposures cannot be classified as carcinogenic to humans.

Anna TOMPA

*Environmental Carcinogens:* Selected Methods of Analysis. Volume 5 — Some Mycotoxins. IARC Scientific Publications No. 44, Lyon 1982, 455 pages. Price: Sw. fr. 60.—

In considering the substances to be included in this manual, first those chemicals were discussed that have been included in the IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. The mycotoxins that have been evaluated in the Monographs were selected on the basis of evidence of human exposure and some experimental evidence of human exposure and some experimental evidence of their carcinogenicity. But several mycotoxins not yet evaluated in the Monographs series have been included also in this volume, including citrinin, mycophenolic acid, rubratoxin, P. islandicum and T2 tricothecene.

Man is exposed to mycotoxins from naturally-occurring contamination of food by parent fungi, or by the use of antibiotic drugs delivered from fungal sources. It was recognized at an early stage of the investigations concerning man, that aflatoxins, although of worldwide occurrence, were found most commonly under hot, humid climates. Many investigations demonstrated that samples of cereals and nuts from markets in tropical countries had impressive levels of contamination. This volume may have been more appropriately entitled "The Aflatoxins and Other Mycotoxins". It is evident that more research and effort has been made in connection with aflatoxins that with other mycotoxins. A review of the more recent investigations of aflatoxins has been included to provide a perspective on the validated methods. Several chapters are included about the significance of the evidence for carcinogenicity of mycotoxins establishing legislative controls on sampling problems and the need for quality control in the laboratory.

Two complementary methods serve the identification of mycotoxins in the environment that may be responsible for human cancer, namely the measurement of specific cancer incidence and the measurement of the occurrence were used for evaluation.

The other mycotoxines dealt with in this volume have received less attention than the aflatoxins, but for some, such as ochratoxin A, fusarenon X and luteoskyrin, there is widespread human exposure and intoxication of domestic animals has been reported.

Patulin was found carcinogenic in rats, but no epidemiological data exist to evaluate its human carcinogenicity. The main surce of patulin in the human diet in apple juice, when apples rotten by Penicillium expansum are included in the processed fruit. Penicillic acid is an antibiotic with demonstrated mutagenecity and unknown chronic toxicity to humans. The carcinogenicity of trichothecenes T-2 toxin is not definitive, although a few studies suggest a carcinogenic action in experimental animals. Luteoskyrin and rubratoxin B are mutagenic to Salmonella typhimurium TM 677 in the presence of an activating enzyme system.

This manual contains selected methods, and particular consideration is given to the requirements of epidemiologists, hygienists and others concerned with the evaluation of carcinogenic and other toxic effects of mycotoxins. Particular consideration is given to biological tests and methods that establish individual past exposure to their environmental presence.

Anna TOMPA

*Environmental Carcinogens Selected Methods of Analysis.* Volume 6 — N-Nitrosamines. Editor-in-Chief: H. EGAN. IARC Scientific Publications No. 45, Lyon 1982. 508 pages. Price: Sw. fr. 80.—

This sixth volume in the series of Manuals of the IARC Scientific Publications represents an updating of a former volume, that on N-nitrosamines published in 1978. The first in the series.

The subject remains one of great importance to the Agency, which has arranged symposia on a regular basis in London (1969), Heidelberg (1971), Lyon (1973), Tallin (1975),

Durham NH (1977), Budapest (1979) and Tokyo (1981). The purpose of these meetings was to facilitate the exchange of up-to-date information between workers in the fields of trace nitrosamine analysis. The last meeting was held in 1983 in Banff, Alberta, Canada.

The subject has developed rapidly in the past five years. These advances are fully reflected in the present volume, which includes the three important sources of human exposure that have become evident: tobacco smoke, endogenous substances and the so-called non-volatile N-nitroso compounds. This volume therefore supplements rather than replaces the first volume in this series.

The manual consists of six main chapters on the analysis of volatile N-nitrosamines in various substrates, e.g. in air, tobacco and tobacco smoke, malt and malt-based bevarages, food and animal feed, biological fluids and in other miscellaneous products. In the other part of the manual 3 chapters deal with the general clean-up and analysis non-volatile N-nitroso compounds: individual substances and substrates. In the analysis of environmental N-nitroso compounds, the main effort is still devoted to the determination of volatile nitrosamines. It is not surprising, therefore, that gas chromatography remains the method most frequently used for their separation and detection. Still, as more emphasis is placed on non-volatile N-nitroso compounds, new chromatographic procedures are being developed and concerned in Chapter IV. Problems are sometimes encountered in the chromatography of volatile nitrosamines, such as a lack of reproducibility and even complete loss of the peaks. As an alternative to gas chromatography, HPLC (high pressure liquid chromatography) is now being used to a greater extent. In general a combination of two detectors would be the most effective approach in nitrosamine separation methods.

Twenty-five years after the carcinogenicity of N-nitrosodimethyl-amine had been demonstrated by Magee and Barnes (1956), scientific research in the field of N-nitroso compounds has virtually exploded. In 1981 more than 1400 papers were published on the analysis, formation, chemistry, biochemistry, metabolism and biological effects of N-nitroso compounds This is probably the main reason to support the publication of this volume of IARC Scientific Publications on *N-nitroso compounds.*

Anna TOMPA

*Tuberculosis control.* Technical Report Series 671. WHO, Geneva 1982. 26 pages.
Price: Sw. fr. 3.—

The present account of a conference held by the joint IUAT/WHO Research Group on the current problems and status of tuberculosis control, Geneva, 14–18 September 1982, falls into 11 sections.

The introduction, Section 1, takes a general look at the subject. It must be recognized that while in the industrialized countries splendid results have been achieved in tuberculosis control (t.c.) over the last three decades, in the developing countries the absolute number of tuberculosis cases has shown a regrettable rise and the epidemiological situation of tuberculosis is very poor. This calls forth the activity of WHO in order to remedy the situation in the developing countries. Section 2 also concentrates on the developing contries where the yearly figures of smear-positive, that is, highly infective, cases amounts to 4 to 5 million, and the incidence of cases of lesser infectivity is nearly the same. In children the pulmonary form of tuberculosis is prevalent. The epidemiological trends also give cause for concern. In the majority of the developing countries the risk corresponds to 2 to 5%, in other words, it is 20 to 25 times as high as in the industrialized countries. The topics of parts 3–5 are: Impact of current control measures; Planning and organization of national tuberculosis programmes; Case-finding. Section 6 is on the laboratory tuberculosis services (control, intermediate, peripheral

laboratory network, culturing, quality control, other laboratory methods). Sections 7–8 deal with chemotherapy, BCG-vaccination and preventive treatment, sections 9–10 with the socio-economic aspects of tuberculosis and point to interesting lines of research under various, i.e. immunological, bacteriological, epidemiological, diagnostic-therapeutic, sociological and health-educational aspects of tuberculosis. In the closing part the conclusions are summed up and recommendations are offered in 11 points. Though these reflect true efforts aimed at optimalization, they leave us with some doubts. In fact, the introduction gives expression to the objective of WHO: "Health for all by the year 2000". On the other hand, the conclusions begin with the statement: "In the hundredth year after Robert Koch's discovery of the tubercle bacillus, tuberculosis is still a major unsolved health problem of world-wide dimension. . .". One may wonder whether the slightly more than 15 years ahead until that date will be sufficient.

L. Cselkó

*Vaccination against Tuberculosis.* Technical Report Series 651. WHO, Geneva 1980. 21 pages
Price: Sw. fr. 2.—

A Scientific Group on Vaccination against Tuberculosis, jointly sponsored by the Indian Council of Medical Research and the World Health Organization, met at the WHO Regional Office for South-East Asia, New Delhi, from 28 April to 2 May, 1980. The aims of the meeting were to review research on BCG vaccination, to assess the present state of knowledge and to determine how this knowledge may be advanced.

The subject-matter of the meeting is presented in the form of 12 chapters and an appendix, in conformity with the traditional arrangement of the other publications in this series.

In chapters 1–5 we find a brief introduction and a thorough coverage of a vaccination trial in South India, involving a population of 360 000 subjects occupying a region west of Madras. The trial was preceded by extensive preliminary studies of multiple aspects centering on the epidemiological characters of the region. (Comments upon these studies, relevant to the subject, form a separate section.) In chapters 6–9 first the various vaccines, and the South-Indian variant of M. tuberculosis are dealt with, subsequently the problems of exogenous reinfection and of immune response are discussed. The questions of nontuberculous mycobacterial infections are condensed in the short but interesting chapter 10. Chapter 11 reports on other studies. Chapter 12 contains the conclusions and recommendations. Hypotheses and recommendations for research are offered in the appendix.

L. Cselkó

*BCG-Vaccination Policies.* Technical Report Series 652, WHO, Geneva 1980. 17 pages.
Price: Sw. fr. 2.—

This publication gives an account of the meeting held by the WHO Study Group on BCG-Vaccination Policies, Geneva, 24–27 June 1980.

In section 1 serving as introduction, the reasons for convening this meeting are given. In fact, certain modifications of the existing BCG-vaccination policies seem necessary in the light of recent knowledge. In section 2–4 the results of earlier investigations are reviewed, particular consideration being given to the trial in South-India, in view of the validity and significance of the results. Section 5 deals with BCG vaccination of neonates and young children. Information on the basic facts and current status of BCG vaccination policies is provided by sections 6 and 7. Section 8 points out the lines of research, section 9 sums up the recommendations of the Committee.

This booklet which ends with a list of 16 references contains all relevant facts in a succinct form, similarly to the earlier WHO publications on the same subject.

L. Cselkó

*Chemotherapy of Leprosy for Control Programmes.* Technical Report Series 675. WHO, Geneva 1982. 33 pages, 1 table. Price: Sw. fr. 4.—

A WHO study group on Chemotherapy of Leprosy for Control programmes met in Geneva from 12 to 16 October 1981. The objectives of the meeting were: 1. to review information collected since 1976 (the year of the last meeting of WHO Expert Committee on Leprosy) on the problems related to the chemotherapy and chemotherapeutic regimen of leprosy;

2. to recommend, for use in leprosy control programmes, appropriate multidrug regimens for multibacillary cases including new, treated and drug-resistant patients, whether clinically suspected or proved;

3. to recommend regimens for paucibacillary cases; and

4. to identify further research needs in the clinical and operational aspects of chemotherapy of leprosy.

The subject-matter of the meeting is given in five chapters, in accordance with the usual arrangement of the reports.

Chapter 1, "The Problem", provides a global review of the secondary and primary resistance to Dapsone, as well as of secondary resistance to other bactericidal antileprosy drugs, the persistence of M. leprae, the difficulties of introduction of therapeutic regimens recommended earlier and of the current status of therapy. Information on drugs for multidrug treatment is found in chapter 2, which deals with the various questions of use of the four main bactericidal antileprosy drugs. Chapter 3 considers the management of multibacillary and paucibacillary leprosy in detail in the context of the recommended therapeutic regimens. Chapter 4, "Operational Aspects", deserves particular interest by covering issues of practical importance, such as health services, detection and registration of cases, screening, laboratory facilities, drug supply, health education, equipment, training of health workers, financial resources, etc. Finally, chapter 5 outlines the trends of future research. The volume closes with a list of 47 references.

L. Cselkó

*Health Care and Traditional Medicine in China, 1800–1982.* Hilliers, S. M., Sewell, J. A. Routledge, Kegan Paul, London–Boston–Melbourne–Henley 1983. 453 pages, 35 plates, 20 figures. Price: £ 25.—

This book gives a fascinating and thorough account of the developments in health care which have taken place in China from 1800 to the present time. It spans all the years from the earliest days of Western missionary medicine, taking the reader through the stormy years of the Nationalist Rule to the foundation of the People's Republic in 1949. It points to the major trends in health care since that time, with due consideration to the attempts to match the objectives in health care to resources available.

The authors who have closely studied the health care system, as well as traditional medicine in China, draw on their wide experience to give an account of traditional medicine, in particular of acupuncture and plant drugs, as well as of the present health care system including prevention, population control, medical education, nutrition and psychiatry. Questions of distribution, decentralization and development are also given consideration.

The impressive scope of the task accomplished by the authors is documented in this remarkable book which, for all its comprehensiveness, confines itself to the essentials and avoids unnecessary details.

Part 1, on the origin and development of health care, spans the past 182 years in the form of five chapters. Chapter 1, "Chinese and Western Medicine in China", 1800–1911, falls into two sections. The first, after a general survey of the period until 1860, discusses various interesting subjects, such as smallpox inoculation, dispensaries in China, arrival of medical missionaries, western medicine, opium trade. The second, taking a look at the period 1860–1911, deals with various topics of interest, such as self-mutilation, infanticide, foot-binding, opium, "teach-preach-and heal", the medical missionary movement, medical education and public health. In Chapter 2, "The Development of Chinese Health Care, 1911–49" we find the following topics: political background; changes in health care; rural and urban health conditions; midwifery; epidemiology, morbidity, major causes of death, public health measures 1911–24, medical education, distribution of resources, the Dingxial (Tingshian) Model, the formation of the Ministry of Health, wartime health services and post-war developments. Chapter 3 bears the title "Health Care and Welfare in China, the 'Ten Great Years' 1959–59". It takes a retrospective look at this period and provides close information on various issues of high interest, to name only the following topics: organisation and financial problems, the "Hundred Flowers" movement, rural health services 1949–58, national plans, collective welfare, traditional medicine. Chapter 4 "The Three Bitter Years: Overture to the Cultural Revolution — Financial and Organisational Perspectives 1960–65", gives a survey of the period after "The Great Leap Forward", covering the following topics: Health care in the countryside, inequalities in money and skilled personnel, effective demand for health care, facilities in the towns, problems in urban health care. Chapter 5 "The Cultural Revolution and After — Health Care 1965–82", falls into two parts. The first refers to the period of the Cultural Revolution, 1965–70, the second to the years 1970–76.

Part 2, "Preventive Health Work in the People's Republic of China, 1949–82", provides a most interesting survey of the various preventive activities of ancient times and modern era, particular consideration being given to the problems of infective diseases and of maternal and child care. Information on preventive health work in the post-Mao-period covers diverse issues, such as industrial medicine, heart disease, environmental pollution.

Part 3, "Traditional Medicine", provides information on the subject, with the aid of numerous illustrations. It includes the following chapters: Theoretical Basis of Chinese Traditional Medicine; Traditional Therapies I.: Acupuncture and Moxibustion; Traditional Therapies II.: Chinese Materia Medica. Chinese Traditional Medicine and Modern Western Medicine. Integration and Separation in China. It is this chapter which gives the closest insight into Chinese Traditional medicine.

Part 4, "Special Topics in Chinese Health Care", deals with various current questions, such as training of medical and paramedical personnel, psychiatry and treatment of mental diseases in China, diet and nutrition, family planning.

The Appendix at the end of the book presents a table of the Chinese Dynasties. There is an excellent index of subjects and names.

L. Cselkó

*The Role of the Health Sector in Food and Nutrition.* Technical Report Series 667. WHO, Geneva 1981. 90 pages. Price: Sw. fr. 6.—

A WHO Expert Committee on the Role of the Health Sector in Food and Nutrition met in Geneva 23 September–1 October 1980.

Part 1 gives introductory information in the usual form. Part 2 points to the extent of the problem, sums up past performance related to the health sector, comments upon the changing concept of health services and considers new opportunities for nutrition promotion. In parts 5–7 the role of health services, of intersectoral activities, of collaboration between the various sectors and of external cooperation in nutrition are evaluated. Part 8 contains the conclusions. The list of 9 references is followed by 8 appendices, all on timely issues of practical interest, such as diagnostic aspects of nutritional deficiency, primary and secondary prevention of nutritional deficiencies, curative and rehabilitative measures (emphasis being laid on protein deficient nutrition in children), administrative structure of nutrition services in the health sector, provision of adequate manpower, integration of nutrition in the curriculum of health workers, optimal methodological use of recent results, research programs providing a basis for health measures. Attention is drawn in various sections of the book to the alarming consequences of deficient nutrition.

L. Cselkó

*Wholesomeness of Irradiated Food.* Technical report series 659, WHO, Geneva 1981. 34 pages. Price: Sw. fr. 3.—

This publication gives an account of the meeting held by the joint FAO/IAEA/WHO Expert Committee on the Wholesomeness of Irradiated Food in Geneva, 27 October–3 November, 1980. The brochure of traditional arrangement falls into 12 parts.

Part 1 contains short introductory notes. Part 2 deals with general aspects and offers explanations. The subsequent parts are in the majority of practical orientation. In parts 3–7 technical, radiochemical, nutritional, microbiological and toxicological issues are dealt with. The implications of irradiation in case of fishes, rice, cocoa-beans, dates, mango, leguminous vegetables and spices are discussed, checking and reevaluation being also considered. Part 10 gives a summary of the acceptability of irradiated food. Part 11 points to future lines of research and offers also recommendations, emphasizing the need for further studies in order to gain closer insight into the radiation effects on the proteins of vegetables and on the biological value of the vitamin-B-complex. (It is to be noted that leguminous vegetables belong to the staple foods in various parts of the world.) The volume closes with a list of 22 references.

L. Cselkó

*Evaluation de certains additifs alimentaires et contaminants.* Technical report series 631, WHO, Geneva 1978. 39 pages. Price: Sw. fr. 5.—

The joint WHO/FAO Expert Committee held its annual meeting in Rome, on 2–11 April, 1978, on the toxicological evaluation of certain food additives and contaminants. The reports and recommendations of the Committee have been summed up in this brochure.

Part 1 serving as introduction outlines the tasks and deals with the regular subjects of the annual meetings, such as evaluation of toxicological studies carried out and being in course, establishment of the respective standards, questions of exposure of the general population, revision of earlier evaluations. One of the most remarkable issues raised for the first time is the use of enzyme preparations and other categories. A further report of high interest deals with long-term exposure ot these agents, in particular in utero and during lactation.

Part 2 defines the microbiological limits, establishes the general lines of toxicological studies and points to general aspects. A separate chapter deals with enzyme preparations,

another with exposure in utero and during lactation. It is in this context that the claim for a coordination of the activities of the various toxicological working groups on the basis of international standards is raised.

Part 3 sets out evaluations and reevaluations. It deals with 9 colouring agents, subsequently with 8 additives, finally with 4 contaminants (asbestos, tin, mercury, lead).

Part 4 revises the standards of certain agents. Part 5 points to future trends of research.

The recommendations are set out in part 6. *1.* The absolute necessity for expert meetings held at least annually, for the evaluation and reevaluation of food additives and contaminants is emphasized by the Committee. *2.* The recommendation of the General WHO Meeting relating to the health hazards of chemicals present in the environment are approved by the Committee. On the other hand, it is stressed that regular evaluation of the food additives and contaminants must be regarded as one of the priorities of WHO. *3.* Priority should be given to the study and evaluation of intentional and non-intentional food additives as well. Setting up of a multidisciplinary FAO/WHO expert committee is, therefore, recommended with the aim of listing all still incompletely clarified chemicals of this kind and of classifying them according to function and potential health hazard. *4.* In the interest of an accelerated evaluation of food additives and contaminants, short-term studies in vitro and in vivo, capable of confirming the suspicion of toxicity, are required. *5.* Studies in neonates for exposure in utero and through maternal milk are of prime necessity. In view of the complexity of the question, organization of a special WHO committee is recommended for this purpose. *6.* Establishment of connections between the various working groups on an international basis should be initiated by WHO. *9.* In the interest of best use of time it is recommended to take off the agenda all those products that are not used as food additives, and FAO is requested to provide for the selection of the chemicals. *8.* Current information should be made available in the future to the Committee by the governments and by the private sector as well.

Appendix 1 contains a list of 44 references. Appendix 2 gives the daily amounts of the various substances tolerable by humans. Appendix 3 lists the chemicals awaiting further toxicological studies.

L. Cselkó

*Evaluation of Certain Food Additives.* Technical Report Series 669, WHO, Geneva 1981. Price: Sw. fr. 3.—

This publication is based on the subject-matter of the annual meeting on the evaluation of certain food additives by the FAO (WHO Expert Committee, Geneva, 1981.). It corresponds in its arrangement to the earlier publications on this subject, only the contaminants receive little attention.

In part 1, the introduction, the program of the Committee is outlined. Part 2 contains 13 topics of high interest and touches upon various problems. After some modifications of the agenda the basic principles of evaluation and specification are dealt with. This is followed by discussions on the importance of the activities of the Committee in the developing countries and on the international connections of these activities. The subsequent part is on the validation of the toxicological data, then the reqirements of technological safety are listed in detail. The problems involved by the use of extracting solvents and enzyme preparations in food industry are also considered. The following topics are: Herbs, spices, and natural food additives; Antibiotics as direct food additives; Hormones in animal production, finally: Plastic materials in food packing.

In part 3, "Comments on Specific Food Additives", food colours, flavouring, sweetening, thickening agents, extraction, carrier-solvents and miscellaneous food additives receive close consideration.

Part 4 revises the specification of 57 additives. Part 5 points to the future tasks of research.

The closing part — part 6 — contains the recommendations of the Committee to FAO/WHO. One of the essential features of these four recommendations is to give priority to the testing of additives for carcinogenic properties.

In Appendix 1, 56 references, the specifications of the substances in question and their daily amounts (mg per kg body-weight) acceptible for human consumption are listed. Appendix 2 and 3 list further substances recommended for toxicological testing. The brochure closes with a short addendum on the specifications of microbial enzyme preparations.

L. Cselkó

*Evaluation of Certain Food Additives and Contaminants.* Technical Report Series 683, WHO, Geneva 1982. 51 pages. Price: Sw. fr. 5.—

This publication includes the subject-matter of the meeting held by the joint FAO/WHO Expert-Committee on Food Additives and Contaminants in Rome, 19–28 April 1982. Its arrangement, identical with that of its predecessors, has become traditional by now.

In the introduction — part 1 — the tasks facing the Committee are pointed out.

Part 2, "General Considerations", discusses ten topics, raising also some new aspects (as for instance the question of a computerized food additive bank). This section includes the following topics: Modification of the agenda; Principles governing the toxicological evaluation of compounds on the agenda; Principles governing the establishment and revision of specifications; Significance of the occurrence of nephrocalcinosis in the toxicological evaluation of modified starches; Food colours extracted from foods; Phosphates and polyphosphates in food additive use; Toxicological evaluation of xenobiotic anabolic agents; Metals occurring in foods; Safety aspects of the Expert Committee and Codex specifications; Food additive computerized data bank.

The most extensive section of the volume, part 3, bears the title "Comments on specific Food Additives and Contaminants". Its topics are arranged into two groups. In the framework of group 1, specific food additives, antioxidants, emulsifying agents, enzymes of microbiological origin used in food processing, flavouring agents, food colours, inorganic salts and buffering agents, sweetening and thickening agents are dealt with. Group 2, on contaminants, is confined to the discussion of metals and xenobiotic agents. This part is of outstanding interest by offering an excellent review of the role of zinc, copper and tin.

Part 4 lists and revises the specifications of various substances. Part 5 outlines the future tasks.

In part 6, recommendations are given. The Committee insists, as before, on the necessity for annual meetings so as to discuss the current problems. Attention is called, among other points, to the phosphates and polyphosphates, to the replacement of calcium by magnesium or other cations, on copper and on the xenobiotic anabolic agents. The implementation of earlier recommendations is evaluated.

As in the earlier publications, Appendix 1 contains references (59 in number). In Appendix 2, specifications and the maximum daily amounts (mg/kg body-weight) of the substances compatible with human use are given. (There are also 24 addenda.) Appendix 3 lists those substances which, according to the Committee, require additional toxicological studies and collection of further information. In Appendix 5 a taxative list of modified starches is found. Appendix 6 provides guidance for evaluation.

L. Cselkó

*Recommended Health Based Occupational Exposure Limits for Selected Vegetable Dusts.* Technical Report Series 684, WHO, Geneva 1983. 78 pages, 2 figures, 8 tables. Price: Sw.fr. 6.—

This is an account of a meeting by a WHO research group in Geneva, 2–8 March 1982. There are 7 chapters.

Chapter 1, though of introductory character, makes the reader acquainted with the essentials of the subject by defining the problem, outlining its extent, providing basic information on the respiratory diseases caused by vegetable dusts, pointing to the current status of exposure limits and their determination, including the "two-step" procedure. Under a separate heading the occupational exposure limits in developing countries are considered and a list of vegetables implying the necessity for the determination of exposure limits is given. Chapter 2 deals with the clinical manifestations in the following order: pathogenesis, pathology, types of response, classification of byssinosis and other respiratory disorders, assessment of individual patients. The topics of chapter 3, all of high interest, are dealt with under their clinical and epidemiological aspects. We find information on byssinosis, including its prevalence all over the world and its clinical course, as also on chronic respiratory diseases and their mortality figures, thorough consideration being given to the particular characters of cotton, flax, hemp and other plantfibres as potential pathogenic factors. Problems of environmental and health assessment are discussed in chapter 4, the effects on health of exposure in chapter 5. Chapter 6 sums up the recommendations of the conference (permissible limits, control, prevention of exposure). Chapter 7 points to the possibilities of future research. This is followed by an impressive list of 165 references. Appendix 1 provides a questionnaire, Appendix 2 lists the pulmonary function tests.

L. Cselkó

*Health Effects of Combined Exposure in the Work Environment.* Technical Report Series 662, WHO, Geneva 1981. 3 tables, 76 pages. Price: Sw. fr. 4.—

The conference held by an expert committee of WHO on the health effects of combined exposures in the work environment, Geneva, 9–15 December 1980, is reported in this publication containing 10 parts.

Part 1, of introductory character, takes a general look at the problems at issue, stating that the importance of the study is based on the fact that workers are often exposed simultaneously or successively to different physical, chemical, biological and psychosocial factors. Furthermore, there are factors such as drug therapy, cigarette smoking, alcohol ingestion and coexistent diseases that may influence individual susceptibility to occupational exposures. Real examples of combined exposure exist in many industrial and agricultural processes. Workers employed in foundries are simultaneously exposed to heat stress, noise, vibration, carbon monoxide, metal fumes and respiratory irritants, in petroleum refineries to a number of solvents, in the cement industry to dust and heat, in welding to metal fumes and many other substances, and in agriculture to pesticides, other chemicals and parasitic diseases. While the effects of individual exposures have been widely studied, there is no uniform approach to combined exposures. Certain basic issues, e.g. types of combined exposure, are dealt with in part 2. Information on the mechanism of responses to combined exposures is provided by part 3. The combined exposures under review are reflected in three types of interaction, those between environmental, biological, physical and chemical agents. In part 4 the relevant investigations, animal experiments and human observations are reviewed. In part 5, personal characteristics influencing responses of combined occupational exposures are dealt with, consideration being given to genetic factors, nutrition and chemical toxicity, drugs, alcohol and smoking, etc.

In chapter 6, the relationships between combined exposure and occupational cancer are examined. Part 7 gives the essential lines of epidemiological approach. Part 8 points to the practical implications of combined exposure, including the necessary tasks. Part 10 sums up the recommendations. A list of 272 references greatly adds to the value of this publication.

L. Cselkó

J. A. H. Waterhouse, C. S. Muir, K. Shaumugaratnam, J. Pavell (eds): *Cancer Incidence in Five Continents.* Vol. IV. WHO, IARC, IACR 1982. 912 pp. Price: Sw. Fr. 100,—

This is the fourth volume of the serial edition of a report on cancer incidence in five continents in a period of 20 years including full registration and statistical evaluation.

The data were contributed by special cancer incidence registering centre from each country. In Hungary these centres presented data from the counties Vas and Szabolcs-Szatmár.

The aim of the report is to study the cancerogenic factors in the environment. It gives exact, comparative information on the differences risk factors in the different areas.

The chapters discuss the following topics: criteria and problems of statistical treatment of the rough data technical details of data registration, problems of tumour classification, methods of coding, techniques of data handling conditions of comparability of data, indices of reliability, the histological typing of cancers, age specific rates of incidence comparison of urban and rural populations, the cumulative rate and the problem of comparability of the data supplied by the different registering centres.

The volume contains a vast amount of multisidedly evaluated data of outstanding importance for practical and theoretical workers in the field of cancer epidemiology.

H. Jellinek

V. S. Turusov (ed.): *Pathology of Tumours in Laboratory Animals.* Volume 3. *Tumours of the Hamster.* International Agency for Research on Cancer, Lyon 1982. Price: Sw. Fr. 80,—

This volume on the tumours of hamsters is the third one in the series. In the previous volumes the tumours of rats and mice were discussed.

The book contains fundamental information on the morphology and classification of the tumours of hamsters. It gives highly useful advice to the experimental pathologist especially on the criteria for making comparable the results of studies on various carcinogens.

The volume covers all tumours of all organs in separate chapters in the following sequence: skin, mammary gland, mouth and cheek-poach, liver, pancreas, respiratory organs, kidney and urinary system, male sex organs, the so-called pigmented costo-vertebrar spot, female sex organs, hypophysis, thyroid gland, adrenals, soft tissues, haemopoetic system, bones and the nervous system. A separate chapter is devoted to the tumours of the djungarian hamster (Phodopus sungorus camobelli).

In each chapter there is a description of the organ's (or system's) normal structure followed by the biology and morphology of the respective tumours both spontaneous and induced. Finally, there is a discussion on comparative features and a list of references.

A special chapter is devoted to the tumours of the soft tissues, to the adenovirus and Rous sarcoma virus induced tumours including ultrastructural and histochemical data.

The light- and electronmicroscopic documentation is of excellent quality.

H. Jellinek

# THE JOHNS HOPKINS MEDICAL INSTITUTIONS

## CLINICAL CYTOPATHOLOGY FOR PATHOLOGISTS
## 1985 POSTGRADUATE INSTITUTE

The Twenty-sixth Postgraduate Institute for Pathologists in Clinical Cytopathology given by The Johns Hopkins University School of Medicine, consists of two courses, both of which must be taken:

— March to May 1985, Home Study Course A is provided each registrant for intensive personal study; and

— May 6 to 17, 1985, In-Residence Course B at the Johns Hopkins Medical Institutions, Baltimore, Maryland, U.S.A.

This Institute is designed solely for pathologists who are Certified (or qualified for certification) by the American Board of Pathology or its international equivalent. It provides an intensive refresher in all aspects of the field of Clinical Cytopathology with time devoted to newer developments and techniques, special problems, and recent applications including immunodiagnosis and needle aspiration. Topics are covered in lectures, explored in small informal conferences, and discussed over the microscope with the Faculty. Abundant self-instructional material is available to augment at individual pace. A loan set of slides with texts (Course A) will be sent to each participant within the United States and Canada for home-study during March and April before Course B in Baltimore. Prior special arrangements to study Course A must be made by participants outside of the United States and Canada. Credit hours 152 in AMA Category I.

*Application* and *completed pre-registration* is advised before *November 30, 1984;* however, completed pre-registration *must* be accomplished *before April 5, 1985*, unless by special arrangement.

For details, write: John K. Frost, M. D., 604 Pathology Building, The Johns Hopkins Hospital, Baltimore, Maryland 21205, U.S.A.

The entire Course is given in *English.*

PRINTED IN HUNGARY
Akadémiai Kiadó és Nyomda, Budapest

# INFORMATION FOR AUTHORS

*Acta Medica Hungarica* is published under the auspices of the Hungarian Academy of Sciences. Manuscripts and editorial correspondence should be sent to the editorial office: H-1450 Budapest 9, P. O. Box 67.

Original articles dealing with clinical and experimental medicine will be accepted with the understanding that they have not been and will not be published elsewhere and are subject to editorial revision.

*Form of manuscripts*

Two copies of the manuscript typewritten double-spaced with margins at least 4 cm wide should be submitted. Pages should be numbered consecutively. The first page should contain (1) the title of the paper (2) the initials and first name(s) of the author(s), (3) name of the institution where the work was done, (4) name and address of the author to whom correspondence and offprint requests should be addressed — this will appear as a footnote; (5) an abstract not exceeding 250 works which states the purposes of the study, the main findings and principal conclusions. Below the abstract provide 3 to 10 keywords that will assist indexers in cross-indexing the article.

The text of the paper should be divided into sections with the headings: Introduction, Materials (Patients) and Methods, Results, Discussion, References.

Unusual abbreviations should be identified in an alphabetical list typed after the abstract and keywords.

Drugs must be referred to by their WHO code designation (Recommended International Nonproprietary Names); use of proprietary names is unacceptable.

The international system of units (SI) should be used for all measurements.

*References*

These should be cited in the text as numbers in square brackets. The list of references should contain in alphabetical order of the first authors' names the following: authors' last names with initials; for journal articles the title of the paper (lower case), journal title abbreviated according to the style used in Index Medicus, volume number, inclusive page numbers, year of publication in parentheses; for books the title (upper and lower case), publisher, place and date of publication. Only manuscripts accepted for publication may be included in the reference list.

Examples:

1. Stagg, B. H., Temperly, J. M., Wyllie, J. H.: The fate of pentagastrin. Gut **12**, 825—829 (1971)
2. Falkner, F.: Prevention in Childhood of Health Problems and Adult Life. WHO, Geneva 1980.
3. Fishman, A. P.: Dynamics of pulmonary circulation. In: Hamilton, W. F., Dow, P. (eds): Handbook of Physiology. American Physiological Society, Washington 1963, pp. 65—79.

*Tables*

Each table should be typed on a separate sheet. They should be numbered consecutively with Roman numerals and have a brief specific title. The data presented in the table must be logically and clearly organized and should be self-explanatory. Omit internal horizontal and vertical rules. Cite each table in the text and indicate its approximate place on the margin.

*Illustrations*

Figures should be submitted in duplicate. They must be numbered consecutively with arabic numerals. All figures should bear the name of the first author, the figure number and an arrow indicating the top. Cite each figure in the text and indicate its approximate place on the margin. If a figure has been published, acknowledge the original source and submit written permission from the copyright holder to reproduce the material. Figure captions should be submitted typed double-spaced on a separate sheet.

*Proofs and reprints*

The first authors will receive (1) comments and suggestions of the Editorial Board for improving their paper; (2) a set of proofs for correction; corrected proofs should be returned without delay to the editorial office; (3) 100 reprints free of charge.

## Periodicals of the Hungarian Academy of Sciences are obtainable at the following addresses:

**AUSTRALIA**
C.B.D. LIBRARY AND SUBSCRIPTION SERVICE
Box 4886, G.P.O., *Sydney N.S.W. 2001*
COSMOS BOOKSHOP, 145 Ackland Street
*St. Kilda (Melbourne), Victoria 3182*

**AUSTRIA**
GLOBUS, Höchstädtplatz 3, *1206 Wien XX*

**BELGIUM**
OFFICE INTERNATIONAL DE LIBRAIRIE
30 Avenue Marnix, *1050 Bruxelles*
LIBRAIRIE DU MONDE ENTIER
162 rue du Midi, *1000 Bruxelles*

**BULGARIA**
HEMUS, Bulvar Ruszki 6, *Sofia*

**CANADA**
PANNONIA BOOKS, P.O. Box 1017
Postal Station "B", *Toronto, Ontario M5T 2T8*

**CHINA**
CNPICOR, Periodical Department, P.O. Box 50
*Peking*

**CZECHOSLOVAKIA**
MAD'ARSKÁ KULTURA, Národní třida 22
*115 66 Praha*
PNS DOVOZ TISKU, Vinohradská 46, *Praha 2*
PNS DOVOZ TLAČE, *Bratislava 2*

**DENMARK**
EJNAR MUNKSGAARD, Norregade 6
*1165 Copenhagen K*

**FEDERAL REPUBLIC OF GERMANY**
KUNST UND WISSEN ERICH BIEBER
Postfach 46, *7000 Stuttgart 1*

**FINLAND**
AKATEEMINEN KIRJAKAUPPA, P.O. Box 128
*SF-00101 Helsinki 10*

**FRANCE**
DAWSON-FRANCE S. A., B. P. 40, *91121 Palaiseau*
EUROPÉRIODIQUES S. A., 31 Avenue de Versailles, *78170 La Celle St. Cloud*
OFFICE INTERNATIONAL DE DOCUMENTATION ET LIBRAIRIE, 48 rue Gay-Lussac
*75240 Paris Cedex 05*

**GERMAN DEMOCRATIC REPUBLIC**
HAUS DER UNGARISCHEN KULTUR
Karl Liebknecht-Straße 9, *DDR-102 Berlin*
DEUTSCHE POST ZEITUNGSVERTRIEBSAMT
Sraße der Pariser Kommüne 3–4, *DDR-104 Berlin*

**GREAT BRITAIN**
BLACKWELL'S PERIODICALS DIVISION
Hythe Bridge Street, *Oxford OX1 2ET*
BUMPUS, HALDANE AND MAXWELL LTD.
Cowper Works, *Olney, Bucks MK46 4BN*
COLLET'S HOLDINGS LTD., Denington Estate
*Wellingborough, Northants NN8 2QT*
WM. DAWSON AND SONS LTD., Cannon House
*Folkstone, Kent CT19 5EE*
H. K. LEWIS AND CO., 136 Gower Street
*London WC1E 6BS*

**GREECE**
KOSTARAKIS BROTHERS INTERNATIONAL BOOKSELLERS, 2 Hippokratous Street, *Athens-143*

**HOLLAND**
MEULENHOFF-BRUNA B.V., Beulingstraat 2,
*Amsterdam*
MARTINUS NIJHOFF B.V.
Lange Voorhout 9–11, *Den Haag*

SWETS SUBSCRIPTION SERVICE
347b Heereweg, *Lisse*

**INDIA**
ALLIED PUBLISHING PRIVATE LTD., 13/14
Asaf Ali Road, *New Delhi 110001*
150 B-6 Mount Road, *Madras 600002*
INTERNATIONAL BOOK HOUSE PVT. LTD.
Madame Cama Road, *Bombay 400039*
THE STATE TRADING CORPORATION OF INDIA LTD., Books Import Division, Chandralok
36 Janpath, *New Delhi 110001*

**ITALY**
INTERSCIENTIA, Via Mazzé 28, *10149 Torino*
LIBRERIA COMMISSIONARIA SANSONI, Via Lamarmora 45, *50121 Firenze*
SANTO VANASIA, Via M. Macchi 58
*20124 Milano*
D. E. A., Via Lima 28, *00198 Roma*

**JAPAN**
KINOKUNIYA BOOK-STORE CO. LTD.
17-7 Shinjuku 3 chome, Shinjuku-ku, *Tokyo 160-91*
MARUZEN COMPANY LTD., Book Department, P.O. Box 5050 Tokyo International, *Tokyo 100-31*
NAUKA LTD. IMPORT DEPARTMENT
2-30-19 Minami Ikebukuro, Toshima-ku, *Tokyo 171*

**KOREA**
CHULPANMUL, *Phenjan*

**NORWAY**
TANUM-TIDSKRIFT-SENTRALEN A.S., Karl Johansgatan 41–43, *1000 Oslo*

**POLAND**
WĘGIERSKI INSTYTUT KULTURY, Marszałkowska 80, *00-517 Warszawa*
CKP I W, ul. Towarowa 28, *00-958 Warszawa*

**ROUMANIA**
D. E. P., *Bucureşti*
ILEXIM, Calea Grivitei 64–66, *Bucureşti*

**SOVIET UNION**
SOJUZPECHAT – IMPORT, *Moscow*
and the post offices in each town
MEZHDUNARODNAYA KNIGA, *Moscow G-200*

**SPAIN**
DIAZ DE SANTOS, Lagasca 95, *Madrid 6*

**SWEDEN**
ALMQVIST AND WIKSELL, Gamla Brogatan 26
*101 20 Stockholm*
GUMPERTS UNIVERSITETSBOKHANDEL AB
Box 346, *401 25 Göteborg 1*

**SWITZERLAND**
KARGER LIBRI AG, Petersgraben 31, *4011 Basel*

**USA**
EBSCO SUBSCRIPTION SERVICES
P.O. Box 1943, *Birmingham, Alabama 35201*
F. W. FAXON COMPANY, INC.
15 Southwest Park, *Westwood Mass. 02090*
THE MOORE-COTTRELL SUBSCRIPTION AGENCIES, *North Cohocton, N. Y. 14868*
READ-MORE PUBLICATIONS, INC.
140 Cedar Street, *New York, N. Y. 10006*
STECHERT-MACMILLAN, INC.
7250 Westfield Avenue, *Pennsauken N. J. 08110*

**YUGOSLAVIA**
JUGOSLOVENSKA KNJIGA, Terazije 27, *Beograd*
FORUM, Vojvode Mišića 1, *21000 Novi Sad*

**Index: 26.015**