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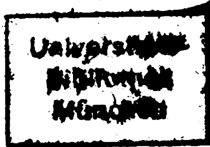

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CONTENTS/INHALTSÜBERSICHT

Reviews/Übersichten

Ebel, H. and Günther, T.
Magnesium metabolism: A review
Magnesium-Stoffwechsel: Eine Übersicht 257

Haeckel, R.
Future perspectives of automatization in clinical chemistry
Zukünftige Perspektiven der Automatisierung in der Klinischen Chemie 455

Marschner, I, Herndl, R. and Scriba, P. C.
Comparison of four different algorithms for the calculation of radioimmunoassay standard curves
Vergleich [illegible] Algorithmen zur Be[illegible] Radioimm[illegible]noa[illegible] Standardkurven 105

Oellerich, M.
Enzyme immunoassays in clinical chemistry: Present status and trends
Enzymimmunotests in der Klinischen Chemie: Gegenwärtiger Stand und Entwicklungstendenzen 197

Tietz, N. W.
Present and future trends in selected areas of clinical enzymology
Gegenwärtige und zukünftige Trends in ausgewählten Gebieten der klinischen Enzymologie 793

Original papers/Originalarbeiten

Agostoni, A., Gerli, Gian Carla, Beretta, L., Bianche, M., Vignali, M. and Bombelli, F.
Superoxide dismutase, catalase and glutathione peroxidase activities in maternal and cord blood erythrocytes
Superoxiddismutase-, Katalase- und Glutathionperoxidase-Aktivitäten in mütterlichen und Nabelschnurblut-Erythrocyten 771

Bachmann, C. and Colombo, J. P.
Determination of orotic acid in children's urine
Bestimmung der Orotsäure im Urin bei Kindern 293

Bartmann, P. und Brendel, W.
Isolierung und Nachweis antigenspezifischer T-Zell-Rezeptoren
Isolation and identification of antigen-specific T-cell-receptors 1

Bencze, K. und Sabatke, L.
Schnelle Phänotypisierung des α_1-Antitrypsins zur Bestimmung der Subtypen des Phänotyps MM
Fast α_1-antitrypsin phenotyping for the differentiation of PiMM-subtypes 13

Benes, P., Morsches, B. und Holzmann, H.
Radioimmunologische Bestimmungsmethoden für Dehydroepiandrosteron und 5-Androsten-3β, 17β-diol
Radioimmunoassay of dehydroepiandrosterone and 5-androsten-3β, 17β-diol 117

Blijenberg, B. G. and Leijnse, B.
A survey report on the determination of total bilirubin in neonatal samples
Bericht über Ringversuche zur Bestimmung des Gesamt-Bilirubins im Serum Neugeborener 27

Bosak, J., Knoll, E., Ratge, D. and Wisser, H.
Single-isotope enzymatic derivative method for measuring catecholamines in human plasma
Enzymatische Einisotopenderivat-Methode zur Bestimmung der Catecholamine im Plasma 413

Breuer, H.
Analytik und biologische Bedeutung der Steroidepoxide
Analysis and biological significance of steroid epoxides 937

Bruckner, A.
Kinetische Glucosebestimmung nach der Glucosedehydrogenase-Methode mit dem Analysenautomaten ACP 5040 (Eppendorf)
The kinetic determination of glucose with the glucose dehydrogenase method using the Eppendorf automatic analyzer 5040 275

Deeg, R., Kraemer, W. and Ziegenhorn, J.
Kinetic determination of serum glucose by use of the hexokinase glucose-6-phosphate dehydrogenase method
Kinetische Bestimmung von Glucose im Serum mit der Hexokinase Glucose-6-phosphat-Dehydrogenase-Methode 49

van Eijk, H. G., van Noort, W. L. Kroos, M. J. and van der Heul, C.
Isolation of the two monoferric human transferrins by preparative isoelectric focussing
Isolierung von Transferrin (Fe) (A) und Transferrin (Fe) (B) des Menschen durch präparative isoelektrische Fokussierung 563

Férard, G., Klumpp, T., Ragagnin, M., Metzger, G. und Métais, P.
Description d'un systeme informatique dans un laboratoire d'analyses biologiques d'urgence
Description of a computer system for the emergency medical laboratory 39

Gaertner, H. J., Wiatr, Gerlinde and Kuss, H. J.
4-methoxy-3-hydroxyphenylglycol as an internal standard for the determination of 3-methoxy-4-hydroxyphenylglycol in urine: results obtained in depressed patients and healthy controls
4-Methoxy-3-hydroxyphenylglykol als interner Standard für die Bestimmung von 3-Methoxy-4-hydroxyphenylglykol im Harn: Ergebnisse bei Patienten mit Depression und gesunden Kontrollpersonen 579

Gärtner, R., Kewenig, M., Horn, K. and Scriba, P. C.
A new principle of thyroxine (T_4) and triiodothyronine (T_3) radioimmunoassay in unextracted serum using antisera with binding optima at extreme pH ranges
Ein neues Prinzip für den Radioimmunassay von Thyroxin (T_4) und Triiodthyronin (T_3) im nicht extrahierten Serum: Verwendung von Antiseren mit Bindungsoptima bei extremen pH-Bereichen 571

Gatschke, W., Gawlik, D. and Kraft, D.
Non-destructive neutron activation analysis of aluminium and phosphorus in bone biopsies
Zerstörungsfreie Neutronen-Aktivierungsanalyse von Aluminium und Phosphor in Knochenbiopsien 403

Gerbitz, K.-D.
Pankreatische B-Zellen-Peptide: Kinetik und Konzentration von Proinsulin, Insulin und C-Peptid in Plasma und Urin, Probleme der Meßmethoden, klinische Aussage und Literaturübersicht
Pancreatic B cell peptides: Kinetic behaviour and concentrations of proinsulin, insulin and C-peptide in plasma and urine, problems of assay methods, clinical significance and literature review 313

Gressner, A. M.
Ribosomal protein modifications in liver injury: Effect of carbon tetrachloride and extrahepatic cholestasis on protein phosphorylation
Ribosomale Proteinmodifikationen bei Leberschädigungen: Einfluß von Tetrachlorkohlenstoff und extrahepatischer Cholestase auf die Proteinphosphorylierung 111

Gressner, A. M.
Evaluation of the assay for serum monoamine oxidase – an index of hepatic fibrosis
Bewertung der Aktivitätsbestimmung der Monoaminoxidase im Serum – ein Parameter der Leberfibrose 921

Gressner, A. M., Köster-Eiserfunke, W., Van de Leur, E. and Greiling, H.
Metabolic and structural studies on serum and liver glycosaminoglycans in normal and liver-injured rats
Metabolische und strukturelle Untersuchungen der Glykosaminoglykane im Serum, im Vergleich zur Leber, bei normalen und leber-geschädigten Ratten 279

Gressner, A. M. und Walraff, P.
Der Einsatz der Lasernephelometrie zur Bestimmung und rechnerunterstützten Auswertung der Fibronectinkonzentration in verschiedenen Körperflüssigkeiten
The use of laser nephelometry for the determination and computer-assisted calculation of the fibronectin concentration in various body fluids 797

Groome, N. P.
Superiority of ABTS over *Trinder* reagent as chromogen in highly sensitive peroxidase assays for enzyme linked immunoadsorbent assay
Überlegenheit von ABTS über *Trinder*'s Reagenz als Chromogen in hochempfindlichen Peroxidase-Bestimmungen für „Enzyme Linked Immunoadsorbent Assay" 345

Gundlach, G. und Mühlhausen, B.
Untersuchungen zur Kupplung des 1-Naphthols mit Fastred-TR
Untersuchungen zur Optimierung einer kontinuierlichen Bestimmung des sauren Phosphatase, 1. Mitteilung
Coupling of 1-naphthol with fast-red-TR
Studies on the optimization of a continuous determination of acid phosphatase, I. 603

Haeckel, R.
Simplified determinations of the "true" creatinine concentration in serum and urine
Vereinfachte Verfahren zur Bestimmung der „wahren" Kreatinin-Konzentration 385

Hajdu, P., Uihlein, M. and Damm, D.
Quantitative determination of clobazam in serum and urine by gas chromatography and fluorometry
Bestimmung von Clobazam in Serum und Urin mittels Gaschromatographie, Dünnschichtchromatographie und Fluorometrie 209

Hansert, E. and Stamm, D.
Determination of assigned values in control specimens for internal accuracy control and for interlaboratory surveys. Evaluation of 200 different lots with identical experimental design: Experiences and conclusions
Sollwert-Ermittlung in Kontrollproben für die laborinterne Richtigkeitskontrolle und für Ringversuche: Erfahrungen an 200 Chargen mit derselben Versuchsanordnung 461

Heimsch, E. und Dobler, T.
Ein Qualitätskontrollsystem für Biotransformationsenzyme in der Dünndarmmucosa
A system for quality control of biotransformation enzymes in the mucosa of small intestine 775

Heinz, F., Pilz, Renate, Reckel, Sylvia, Kalden, J. R. and Haeckel, R.
A new spectrophotometric method for the determination of 5'-nucleotidase
Eine neue spektrophotometrische Methode zur Bestimmung von 5'-Nucleotidase 781

van Helden, W. C. H., van der Slik, W., Persijn, J.-P. and Souverijn, J. H. M.
Automated method for the determination of 5'-nucleotidase in serum by continuous flow analsis
Automatisches Verfahren zur Ermittlung der 5'-Nucleotidase-Aktivität im Serum mit kontinuierlicher Durchflußanalyse 333

Hinghofer-Szalkay, H. und Wurm, H.
Der Einfluß von Hämolyse auf Dichtemessungen an Kapilarblutplasma mittels der Biegeschwingermethode
The influence of haemolysis on capillary blood plasma density measurements using the mechanical oscillator technique 615

Hoffmann, G. E., Blum, K. and Weiss, L.
Overestimation of glucose in uranyl acetate-treated samples: Modification of the deproteinization method
Überhöhte Glucosewerte in Uranylacetat-behandelten Proben: Modifikation der Enteiweißungsmethode 129

Honecker, H., Coper, H., Fähndrich, Christa and Rommelspacher, H.
Identification of tetrahydronorharmane (tetrahydro-β-carboline) in human blood platelets
Nachweis von Tetrahydronorharman (Tetrahydro-β-carbolin) in menschlichen Thrombocyten 133

Hubsch, G., Houot, O. and Henry, J.
Influence of turbidity on photometric assays: A blank sample must always be used
Einfluß der Trübung auf photometrische Bestimmungen: Muß jeweils ein Proben-Leerwert benutzt werden? 149

Ideo, G., Tempini, Silvana, Bellobuono, A., Bellati, G. and Ronchi, G.
Biochemical and histochemical estimations of liver γ-glutamyltransferase activity in ethanol-fed rats
Biochemische und histochemische Untersuchung der Aktivität von γ-Glutamyltransferase in der Leber Ethanolgefütterter Ratten 237

Ikeda, M., Nanba, N., Hayakawa, S. and Ohmori, S.
Colorimetric determination of glycine conjugates of bile acids
Kolorimetrische Bestimmung von Glycin-Konjugaten von Gallensäuren 407

Jäckel, W., Heimes, J. und Herrmann, J.
Der Einfluß des Funktionszustands der Schilddrüse auf den Anstieg des cyclischen AMP im Plasma nach Glukagoninjektion
The effect of thyroid function on the increase of plasma cyclic AMP following glucagon injection 909

Jüppner, H., Mohr, H. and Hesch, R.-D.
Adsorption of parathyrin: pitfall for solid phase assays using radiolabelled antibodies?
Adsorption von Parathyrin: Werden dadurch Solid-Phase-Assays in Frage gestellt? 585

Kälberg, M. and Tobiasson, P.
Determination of cholic and chenodeoxycholic acid in serum: Evaluation of two commercial radioimmunoassay methods
Bestimmung von Cholsäure und Chenodesoxycholsäure im Serum: Bewertung zweier käuflicher Radioimmunassay-Methoden 491

Kahn, S. N., Shortman, R. C., Khan, R. A. and Thompson, E. J.
Effect of sample preparation on cerebrospinal fluid protein patterns in polyacrylamide gels
Effekt der Probenvorbereitung auf das Proteinmuster von Liquor in Polyacrylamidgelen 23

Kamarýt, J. and Matýsěk
β-Glucuronidase activity in amniotic fluid
Die Aktivität der β-Glucuronidase im Fruchtwasser 611

Kaulhausen, H., Rohner, H. G., Siedek, M., Lafosse, M. und Breuer, H.
Bindungskapazität von sexualhormon- und corticosteroid-bindendem Globulin im Serum bei Männern mit Lebercirrhose
Binding capacity of sex hormone binding globulin and corticosteroid binding globulin in serum of male patients with liver cirrhosis 137

Kleesiek, K., Czernik, A. und Eberhard, A.
Klinisch-chemische Diagnostik depressiver Syndrome mit Hilfe eines neuroendokrinen Funktionstestes
Clinical chemical diagnosis of affective diseases by means of a neuroendocrine function test 867

Kleine, T. O.
Liquordiagnostik: Untersuchungen mit Schnelldiagnostica. Untersuchungen zur Adsorption von Proteinen in Glas- und Kunststoffröhrchen
Diagnosis of cerebrospinal fluid: The use of semiquantitative rapid tests. Investigation of the adsorbance of proteins to glass and plastic tubes 7

Kleine, T. O. and Merten, B.
Rapid manual immunoturbidimetric and immunoephelometric assays of prealbumin, albumin, IgG, IgA and IgM in cerebrospinal fluid
Schnelle manuell-immunoturbidimetrische und immunonephelometrische Tests für Präalbumin, Albumin, IgG, IgA, und IgM im Liquor cerebrospinalis 245

Knoll, E., Wisser, H. und Rautenstrauch, H.
Vergleichende Untersuchung zur diagnostischen Wertigkeit von Diskelektrophorese der Urinproteine und N-Acetylglucosaminidaseausscheidung zur Erkennung von tubulären Nierenschädigungen bei chronischer Polyarthritis
Comparative study of the diagnostic value of disc electrophoresis of urinary proteins and measurement of the excretion of N-acetylglucosaminidase for the detection of renal tubule damage in chronic polyarthritis 53

Koch, C.-D., Arnst, E. und Rommel, K.
Harnstoff, Kreatinin, Harnstoff- und Kreatinin-Clearance: Untersuchungen an 25 gesunden Probanden über ein Jahr
Urea and creatinine levels and clearances: Observations in 25 healthy subjects for one year 423

Koch, G., Johansson, U. and Arvidsson, E.
Radioenzymatic determination of epinephrine, norepinephrine and dopamine in 0.1 ml plasma samples
Plasma catecholamine response to submaximal and near maximal exercise
Radioenzymatische Bestimmung von Adrenalin, Noradrenalin und Dopamin in 0.1 ml Plasma-Proben. Plasma-Katecholaminkonzentrationen und Plasma-Renin-Aktivität in Ruhe und bei submaximaler und nahezu maximaler Belastung 367

Külpmann, W. R.
Eine gaschromatographische Methode zur Bestimmung von Carbamazepin, Phenobarbital, Phenytoin und Primidon im gleichen Serumextrakt
A gas-chromatographic method for the determination of carbamazepine, phenobarbital, phenytoin and primidone in the same extract of serum 277

Külpmann, W. R.
Eine gaschromatographische Methode zur gleichzeitigen Bestimmung von Ethosuximid und Valproinat im Serum
A gaschromatographic method for the simultaneous determination of ethosuximide and valproate in serum 339

Lang, C., Gyr, K., Kraehenmann, J. A. and Arenz, F.
The oral pancreatic function test with N-benzoyl-*L*-tyrosyl-*p*-aminobenzoic acid: acute toxicity and effects of renal function on this test
Der orale Pankreas-Funktions-Test mittels N-Benzoyl-*L*-tyrosyl-*p*-aminobenzoesäure: Akute Toxizität und Beeinflussung durch die Nierenfunktion 551

Lanser, A., Blijenberg, B. G., Wallenburg, H. X. S. and Leijnse, B.
Evaluation of an enzyme-immunoassay for the determination of total serum estrogens in pregnancy
Evaluation eines Enzymimmunoassay zur Bestimmung der Gesamt-Östogene im Serum während der Schwangerschaft 327

Lauber, K.
Bestimmung von Eisen im Serum: Methodenvergleich Teepol/Dithionit/Bathophenanthrolin gegen Guanidin/Ascorbinsäure/Ferrozin
Determination of serum iron; a comparison of two methods: Teepol/dithionite/bathophenanthroline versus guanidine/ ascorbic acid/Ferrozine 147

Lindwall, L., Molin, L. and Bergdahl, B.
An evaluation method providing confidence intervals applied to radioimmunoassay
Anwendung einer Auswertungsmethode zur Gewinnung von Vertrauensbereichen auf Radioimmunassays 879

Mann, K., Göring, W., Lipp, W., Keipert, B., Karl, H. J., Geiger, R. and Fink, E.
Radioimmunoassay of human urinary kallikrein. Determination of human urinary kallikrein, II
Radioimmunoassay für Kallikrein im Harn des Menschen. Bestimmung von menschlichem Harnkallikrein, II 395

Milano, G., Schneider, M., Cambon, P., Bouglil, J. L., Barbe, J., Renee, N. and Lalanne, C. M.
An improved method for routine analysis of polyamines in biological fluids with a conventional amino acid analyzer
Verbesserte Methode zur Routine-Bestimmung von Polyaminen in biologischen Flüssigkeiten mit einem konventionellen Aminosäure-Analysator 157

Mordasini, R. C. and Riesen, W. F.
Electroimmunoassay and radioimmunoassay for the quantitation of high density apolipoproteins A-I and A-II
Vergleich zwischen Elektroimmunoassay und Radioimmunoassay zur Bestimmung der Apolipoproteine A-I und A-II 917

Moser, M., Hinghofer-Szalkay, H., Kenner, Th. und Holzer, H.
Die Bestimmung des kolloidosmotischen Drucks aus der Plasmadichte mittels der Biegeschwingermethode
Measurement of the colloid osmotic pressure from plasma density by means of the mechanical oscillator technique 233

Müller-Matthesius, R. und Opper, C.
Der Einfluß von Meßzeit und Reaktionsmilieu bei kinetischen immunturbidimetrischen Proteinbestimmungen
Influence of measurement time and reaction medium on kinetic immunturbidimetric protein determination 501

Naus, A. J., Borst, A. and Kuppens, P. S.
The use of patient data for the calculation of reference values for some haematological parameters
Die Verwendung von Patienten-Daten für die Ermittlung von Referenzwerten für einige hämatologische Kenngrößen 621

Newman, R. A., Fricke, U., Klein, P. J., Uhlenbruck, G. and De Vries, A. L.
Studies on the fate of defined asialoglycoproteins in the circulation of the mouse
Untersuchungen über das Schicksal definierter Asialoglykoproteine im Kreislauf der Maus 31

Nocke-Finck, Lieselotte, Breuer, H. und Reimers, D.
Wirkung von Rifampicin und Streptomycin auf die Konzentrationen von Testosteron und Cortisol im Blut von Männern
Effects of rifampicin and streptomycin on the concentrations of testosterone and cortisol in the blood of men 897

Oellerich, M., Engelhardt, P., Schaadt, M. and Diehl, V.
Determination of methotrexate in serum by a rapid, fully mechanized enzyme immunoassay (EMIT)
Bestimmung von Methotrexat im Serum mit einem rasch durchführbaren, vollmechanisierten Enzymimmunotest (EMIT) 169

Oldendörp, J. und Reinauer, H.
Der Einfluß von Insulin auf den Stoffwechsel von isolierten Glomerula der Ratte
The effect of insulin on the metabolism of isolated rat glomeruli 901

Olek, K., Uhlhass, S. und Wardenbach, P.
Quantitative Bestimmung der *o*-Phthaldialdehyd-Derivate von Noradrenalin, Dopamin und Normetanephrin aus dem Urin mit einem Aminosäureanalysator
Quantitative determination of *o*-phthaldialdehyde derivatives of noradrenaline, dopamine and normetadrenaline in urine with an amino acid analyzer 567

Paar, D. and Maruhn, D.
Spectrometric determination of urokinase in urine after gel filtration, using the chromogenic substrate S-2444
Spektrometrische Bestimmung von Urokinase mit dem chromogenen Substrat S-2444 im Harn nach Gelfiltration 557

Pott, G., Meyering, M., Voss, B., Karges, H. E. and Sieber, A.
Rapid determination of fibronectin by laser nephelometry. Fibronectin concentrations in plasma in human diseases, I
Schnelle Bestimmung von Fibronectin durch Lasernephelometrie; Fibronectinkonzentrationen im Plasma bei Erkrankungen des Menschen, 1. Mitteilung 893

Puukka, M., Puukka, Raija and Reunanen, M.
A rapid and simple gas-liquid chromatographic determination of valproic acid (α-propylvaleric acid) in serum
Schnelle und einfache gaschromatographische Bestimmung von Valproinsäure (α-Propylvaleriansäure) im Serum 497

Sambasiva, J. and Rao, V. H.
Urinary excretion of collagen metabolites in protein malnutrition
Ausscheidung von Kollagen-Metaboliten im Harn bei Protein-Mangelernährung 287

Reiber, H.
Eine schnelle und einfache nephelometrische Bestimmungsmethode für Protein im Liquor cerebrospinalis
A rapid and simple nephelometric assay for total protein in cerebrospinal fluid 123

van Rijn, H. J. M., Boer, P. and Klosse, J. A.
The determination of acid phosphatase of prostatic origin with the automatic clinical analyzer (ACA, DuPont)
Die Bestimmung der sauren Prostata-Phosphatase mit dem Automatic Clinical Analyzer (ACA, DuPont) 627

Schena, F. P., Manno, C., D'Agostino, R., Bruno, G., Cramarossa, F. and Bonomo, L.
A kinetic test for the assay of the C1-esterase inhibitor
Kinetischer Test für die Bestimmung des C1-Esterase-Inhibitors 17

Schlebusch, H., Sorger, M., Munz, E., Kessler, A.-Ch. und Zwez, W.
Glucosebestimmung in hämolysierten Blutproben
Determination of glucose in hemolysed blood samples 885

Schmitz-Huebner, U., Nachbar, J. and Asbeck, F.
The determination of antithrombin III, α_2-macroglobulin and α_2-antiplasmin in plasma by laser nephelometry
Die Bestimmung von Antithrombin III, α_2-Makroglobulin und α_2-Antiplasmin im Plasma mittels Lasernephelometrie 221

Schön, F., Hackenberg, K., Paar, D. und Reinwein, D.
Die Bestimmung von Progesteron im Harn mit der kompetitiven Proteinbindungsmethode
Determination of progesterone in urine by a competitive protein binding method 355

Scholer, A., Pianezzi, A., von Rickenbach, R. und Vonderschmitt, D. J.
Verbesserte Methode zur Bestimmung der Harnsäure auf dem SMAC®
An improved method for the determination of uric acid on a SMAC® analyzer 363

Schramel, P., Wolf, A. und Klose, B.-J.
Direktbestimmung von Aluminium in Serumproben mittels Inductively Coupled Plasma (ICP)-Emissionsspektralanalyse
Determination of aluminium in blood-serum by Inductively Coupled Plasma (ICP)-Spectroscopy 591

Schwarz, S.
Radioimmunoassay evaluation and quality control by use of a simple computer program for a low cost desk top calculator
Auswertung und Qualitätskontrolle von Radioimmunoassays mit einem einfachen Programm für einen kostengünstigen Tischrechner 215

Schwörer, Ina und Kaul, A.
Tierexperimentelle Untersuchungen über die Elimination von inkorporiertem Blei unter biologischen Streßsituationen und Applikation von Na_2Ca-EDTA
Elimination of lead from rats under biological stress, and application of Na_2Ca-EDTA 163

Staab, H. J., Anderer, F. A., Stumpf, E. und Fischer R.
Die prognostische Bedeutung von zirkulierenden Immunkomplexen des Carcinoembryonalen Antigens (CEA) bei Patienten mit Adenokarzinomen des Gastrontestinaltraktes
Prognostic value of circulating immune complexes of carcinoembryonic antigen (CEA) in patients with adenocarcinoma of the gastrointestinal tract 175

Staiger, Ch., de Vries, J. and Walter, E.
A rapid and sensitive method for the determination of phenazone (antipyrine®) using gas-liquid-chromatography with nitrogen detection
Eine einfache und empfindliche Methode für die Bestimmung von Phenazon (Antipyrin®) mittels Gas-Chromatographie mit Stickstoffdetektor 817

Tel, R. M. and Berends, G. T.
Incomplete hydrolysis of cholesteryl esters during the enzymatic cholesterol determination as evidenced by aqueous cholesteryl ester solutions: Comparison of six enzymatic procedures with the *Liebermann-Burchard* method
Unvollständige Hydrolyse von Cholesterinestern bei der enzymatischen Cholesterinbestimmung in wäßrigen Lösungen von Cholesterinestern: Vergleich von sechs enzymatischen Verfahren mit der *Liebermann-Burchard*-Methode 595

van Thiel, Dagmar, Marschner, I., Wood, W. G., Habermann, J. und Scriba, P. C.
Methodische Untersuchungen und Vergleich von 7 kommerziellen Kits zur radioimmunologischen Thyrotropin-Bestimmung aus dem getrockneten Blutstropfen
Development of a radioimmunoassay for thyrotropin (TSH) in dried blood spots together with a comparison of 7 commercial kits 807

Tomcsányi, A., Fekete, B., Schweiger, O. und Kertész, L.
Unterschiede für Immunglobulin E im Serum, bestimmt mit kompetitiven bzw. Papier-Radioimmunosorbenttest: Beseitigung durch Vorbehandlung mit Polyethylenglycol
Differences in the levels of serum immunoglobulin E determined by the competitive radio-immuno-sorbent and the paper-radio-immuno-sorbent methods: Removal of these differences by pretreatment of sera with polyethylene glycol 145

Unger, Th. and Struck, H.
Hydrolysis of some cystine aminopeptidase and aminopeptidase substrates by human placental enzymes after isoelectric focusing
Hydrolyse einiger Substrate für Cystinaminopeptidase und Aminopeptidase durch Enzyme aus der Placenta vom Menschen nach isoelektrischer Fokussierung 631

Verheesen, P. E., Brombacher, P. J., Cremers, H. M. H. G. and de Boer, R.
Determination of low levels of bupivacaine (Marcaine®) in plasma during epidural analgesia
Bestimmung von niedrigen Bupivacain (Marcain®)-Konzentrationen im Plasma während Epiduralanalgesie 351

Wilhelm, A., Berge, P.-G. und Schriewer, H.
Zur Aktivitätsbestimmung der Sialyltransferase im menschlichen Serum
Analysis of sialyltransferase activity in human serum 271

Wisser, H., Knoll, E. und Schmid, G.
Tageszeitliche Änderungen erhöhter Aktivitäten der sauren Phosphatase beim Prostatacarcinom
Diurnal variation of the elevated acid phosphatase activity in cases of prostate carcinoma 297

Wood, W. G., Bauer, M., Horn, K., Marschner, I., van Thiel, Dagmar, Wachter, Christine and Scriba, P. C.
A second external quality control survey (EQCS) for serum triiodothyronine (T_3) and thyroxine (T_4) assays using the "Munich model"
Ein zweiter Ringversuch für Triiodthyronin (T_3) und Thyroxin (T_4) im Serum 511

Wood, W. G., Bauer, M. Marschner, I. and Scriba, P. C.
An external quality control survey (EQCS) for serum cortisol
Externer Ringversuch für Cortisol im Serum 183

Wood, W. G., Butz, Rosemarie, Casaretto, Monika, Hehrmann, R., Jüppner, H., Marschner, I., Wachter, Christine, Zahn, H. and Hesch, R. D.
Preliminary results on the use of an antiserum to human parathyrin in a homologous radioimmunoassay
Erste Ergebnisse mit einem Antiserum gegen Parathyrin vom Menschen für einen homologen Radioimmunassay 789

Zaugg, H.
Thromboplastic activity of human arterial walls and its interaction with the plasmatic coagulation system
Thromboplastin-Aktivität in humanen arteriellen Gefäßwänden und ihre Wirkung auf das plasmatische Gerinnungssystem 545

Zazgornik, J., Kopsa, H., Schmidt, P., Pils, P., Balcke, P., Hysek, H. und Deutsch, E.
Konzentration von α_1-Antitrypsin im Serum dialysierter und nierentransplantierter Patienten
α_1-Antitrypsin level in serum of dialyzed and renal transplant patients 241

Zoppi, F., Peracino, A., Marcovina, S. and Fenili, D.
Development and improvement of a commercial uric acid enzymatic determination kit on a centrifugal fast analyzer
Entwicklung und Verbesserung eines kommerziellen Testbestecks zur enzymatischen Harnsäurebestimmung an einem Zentrifugalanalysator 59

Short communications/Kurzmitteilungen

Dick, W.
Glucosebestimmung in Venen- und Kapillarblut mit der Glucose-dehydrogenase-Methode in einer neuen Hämolysierlösung am Auto Analyzer II
A new hemolysing agent for the determination of blood glucose (venous and capillary) by the glucose dehydrogenase method in the Autoanalyzer II 255

Nagel, Marianne and Schümann, H.-J.
A sensitive method for determination of conjugated catecholamines in blood plasma
Eine empfindliche Methode zur Bestimmung der konjugierten Katecholamine im Plasma 431

Rastogi, S. C., Clausen, J., Melchior, J. C. and Dyggve, H. V.
Abnormal serum α_2-macroglobulin in *Dyggve-Melchior-Clausen* syndrome
Abnormales α_2-Makroglobulin im Serum bei *Dyggve-Melchior-Clausen*-Syndrom 67

van der Sluijs Veer, G.
Referenzwerte für die Lactatdehydrogenase im Serum im Kindesalter und in der Pubertät
Reference values for lactate dehydrogenase in the serum during childhood and puberty 305

Sutor, D. June and Wilkie, Lynette I.
Measurement of free palmitic acid in human bile
Bestimmung der freien Palmitinsäure in der Galle von Menschen 193

Uldall, A.
Stable reagents and a suitable routine procedure for the cystinuria test
Stabile Reagenzien und ein geeignetes Verfahren für den Test auf Cystinurie 303

Guest Editorial

Whitehead, T. P.
Future strategies in clinical chemistry
Zukünftige Strategien in der Klinischen Chemie 453

Editorial

Uhlenbruck, G.
Lektine: Einführung, Entwicklungen und Aussichten
Lectins: Introduction, trends and perspectives 373

ECCLS Communication/ECCLS-Kommunikation

ECCLS
European Committee for Clinical Laboratory Standards
Revised Byelaws
Revidierte Satzung (*in Englisch*) 637

Abstracts/Autorenreferate

2nd Symposium on Lectine, December 14, 1979, Cologne
2. Lektin-Symposium am 14. Dezember 1979, Köln 375

Joint Congress of the Scandinavian and German Societies of Clinical Chemistry
Gemeinsamer Kongreß der Skandinavischen und Deutschen Gesellschaften für Klinische Chemie 647

Workshop Conferences of the German Society for Clinical Chemistry
Kleinkonferenzen der Deutschen Gesellschaft für Klinische Chemie

Haeckel, R.
Statistische Probleme beim Vergleich von klinisch-chemischen Analysen-Verfahren
Bericht über die Kleinkonferenz der Deutschen Gesellschaft für Klinische Chemie am 28. und 29. Juni 1979 in Hannover
Statistical problems in the comparison of methods of clinical chemical analysis
Report on the workshop conference of the German Society for Clinical Chemistry held on June 28 and 29, 1979 in Hannover 433

Rao, G. S. and Breuer, H.
UDP-Glucuronyltransferase
Report of the workshop conference of the German Society for Clinical Chemistry held on September 27–28, 1977 in Schloß Auel
UDP-Glucuronyltransferase
Bericht über die Kleinkonferenz der Deutschen Gesellschaft für Klinische Chemie am 27. und 28. September 1977 in Schloß Auel 307

Wagener, C. und Breuer, H.
Diagnostische Bedeutung von Tumormarkern in der Klinischen Chemie
Bericht über die Kleinkonferenz der Deutschen Gesellschaft für Klinische Chemie vom 15.–17. November 1979 in Schloß Auel
Diagnostic significance of tumour markers in clinical chemistry
Report on the workshop conference of the German Society for Clinical Chemistry, held on November 15–17, 1979 in Schloß Auel 821

IFCC Sections

IFCC Section (1979) no. 3
Büttner, J., Borth, R., Boutwell, J. H. Broughton, P. M. G. and Bowyer, R. C.
Approved recommendation (1978) on quality control in clinical chemistry
Part 1: General principles and terminology 69
Part 2: Assessment of analytical methods for routine use 78

Bowers, G. N. Jr., Bergmeyer, H. U. Hørder, M. and Moss, D. W.
IFCC Methods for the measurement of catalytic concentration of enzymes
Part 1: General considerations concerning the determination of the catalytic concentration of an enzyme in the blood serum or plasma of man 89

Hobbs, J. R., Harboe, N., Alper, C., Johansson, B. G. and Peters, Th.
Provisional recommendation (1978) on specification for human serum albumin standard 96

Hobbs, J. R., Harboe, N., Alper, C. and Johansson, B. G.
The human serum standard IFCC 74/1 99

IFCC Section (1980) no. 1
Porter, C. J. and Curnow, D. H. (IFCC 1980/1)
Provisional recommendation (1979) on a scheme for a two year postgraduate course in clinical chemistry 439

Haeckel, R., Collombel, Ch., Geary, T. D., Mitchell, F. L., Nadeau, R. G. and Okuda, K. (IFCC 1980/2)
Provisional guidelines (1979) for listing specifications of spectrometers in clinical chemistry 445

IFCC 1980/3
Bergmeyer, H. U. and Hørder, M.
IFCC Methods for the measurement of catalytic concentration of enzymes, Part 3. IFCC method for alanine aminotransferase 521

IFCC 1980/4
Büttner, J., Borth, R., Broughton, P. M. G. and Bowyer, R. C.
Quality control in clinical chemistry, Part 4. Internal quality control 535

IFCC 1980/5
IFCC Document with a view to a Recommendation:
Siggaard-Andersen, O., Durst, R. A. and Maas, A. H. J.
Physico-chemical quantities and units in clinical chemistry with special emphasis on activities and acitivity coefficients 829

IFCC 1980/6
Büttner, J., Borth, R., Boutwell, J. H., Broughton, P. M. G. and Bowyer, R. C.
Approved recommendation (1979) on quality control in clinical chemistry, Part 3. Calibration and control materials 855

IFCC 1980/7
Büttner, J., Borth, R., Boutwell, J. H., Broughton, P. M. and Bowyer, R. C.
Approved recommendation (1979) on quality control in clinical chemistry, Part 6. Quality requirements from the point of view of health care 861

IFCC 1980/8
IFCC Document with a view to a Recommendation
Okuda, K.
Provisional guidelines (1980) for listing specifications of clinical chemical analysers 947

Honours/Ehrungen

Honorarprofessur für W. Bürgi (Aarau)
Honorary Professorship for W. Bürgi (Aarau) 437

Senior Award der National Academy of Clinical Biochemistry für J. Büttner
Senior Award of the National Academy of Clinical Biochemistry for J. Büttner 635

Verleihung der Scherer-Medaille an Hansjürgen Staudinger
Presentation of Scherer Medal to Hansjürgen Staudinger 929

Acknowledgement
Danksagung 954

Errata

In the section Terminology (p. 75) of IFCC Section (1979) no 3, Approved Recommendation (1978) on Quality Control in Clinical Chemistry, Part I – General Principles and Terminology, this J. *18*, 69–77 (1980), the paragraph *Method, definitive* should read:
Method, definitive. A method, which after exhaustive investigation is found to have no known source of inaccuracy or ambiguity.

In the paper by Heinemann, G., Löschenkohl, K. and Schievelbein, H., this J., 17, 647–651 (1979) the last sentence of the paragraph "Preparation of HbCO-containing blood samples" should read: "However, it should be borne in mind that it is difficult to obtain nominal values by mixing parts of O_2- and CO-saturated blood, because HbO_2 is changed to HbCO by physically dissolved carbon monoxide (2)."

In the abstract 4.15 by Blossey, H.-Ch. et al., this J. *18*, 729–730 (1980) the heading of the third block of table 1 should read:
"Cold" MPA, after
[^{3}H]DES [^{3}H]R 5020 [^{3}H]R 1881

Book reviews/Buchbesprechungen

Adrian, R. H. et al.
Reviews of Physiology, Biochemistry and Pharmacology 594

Arber, W. et al.
Current Topics in Microbiology and Immunology, vol. 78 104

Auer, L. M.
The Pathogenesis of Hypertensive Encephalopathy 542

Bessis, M., Shohet, S. B. and Mohandas, N.
Red Cell Rheology 196

Beyermann, K.
Chemie für Mediziner, 4. Aufl. 196

Blauer, G. und Sund, H.
Transport by Proteins 544

Bloemendal, H.
Journal "Molecular Biology Reports". Special EMBO Issue on RNA and RNP. Vol. 5, No. 1/2 543

Breddin, H. K.
Prostaglandine und Plättchenfunktion – Methoden zur Erfassung einer gesteigerten Plättchenfunktion. Chromogene Substrate im Gerinnungslaboratorium 195

Brock, T. D.
Thermophilic Microorganisms and Life at High Temperatures 452

Brück, K. et al.
Examens-Fragen Physiologie, 4. Aufl. 452

Daldrup, T.
Postmortaler Eiweißzerfall in menschlichen Organen. Reaktionen und zeitliche Zusammenhänge 543

Dargel, R. und Graf, W.
Lehrprogramme der funktionellen Biochemie, Bd. 3, 4 und 5 450

Doss, M.
Diagnosis and Therapy of Porphyrias and Lead Intoxication 104

Dünges, W.
Prächromatographische Mikromethoden 761

Ebel, S.
Synthetische Arzneimittel 451

Eisenberg, S.
Progress in Biochemical Pharmacology, vol. 15. Lipoprotein Metabolsim 195

Fishbein, L.
Potential Industrial Carcinogens and Mutagens 542

Frunder, H.
Effects and Metabolism of Insulin and Cyclic Nucleotides 761

Haschen, R. und Scheuch, D.
Abriß der Pathologischen Biochemie 542

Kelley, W. N. and Weiner, I. M.
Uric Acid. Handbook of Experimental Pharmacology, vol. 51 594

Labhart, A.
Klinik der inneren Sekretion, 3. Aufl. 544

Lindauer, M., Nydegger, U. E., Matern, S. and Gerok, W.
Reviews of Physiology, Biochemistry and Pharmacology, vol. 85 544

Lohmann, K.
Ergebnisse der experimentellen Medizin, Bd. 32 452

Marx, R. und Thies, H. A.
Niere, Blutgerinnung und Hämostase. XXI. Hamburger Symposium über Blutgerinnung 450

Matthies, D.
Biochemische Formelsammlung 196

Natelson, S., Pesce, A. J. and Dietz, A. A.
Clinical Immunochemistry. Chemical and Cellular Bases and Applications in Disease 543

Pungor, E.
Coulometric Analysis 761

Schwiegk, H.
Handbuch der inneren Medizin. II/6 Blut und Blut und Blutkrankheiten, 5. Aufl. 594

Short, R. V.
British Medical Bulletin, vol. 35, No. 2. Reproduction 761

Svejgaard, A. et al.
Monographs in Human Genetics. The HLA System – An Introductory Survey, 2nd Edition 543

Thielmann, K., Himmler, V., Köhler, W. und Stein, J.
Wörterbuch der Biochemie. Deutsch–Englisch–Französisch–Russisch–Spanisch 451

Vahlensieck, W. und Gasser, G.
Pathogenese und Klinik der Harnsteine VI 451

Young, Donald S., Uddin, D., Nipper, H., Hicks, J. and King, J. Stanton
Clinician and Chemist, The Relationship of the Laboratory to the Physician, Proceedings of the First Arnold O. Beckmann Conference in Clinical Chemistry 195

Buchhinweise/Book indications

Adrian, R. H., Helmreich, E., Holzer, H., Jung, R., Krayer, O., Linden, R. J., Lynen, F., Miescher, P. A., Piiper, J., Rasmussen, H., Renold, A. E., Trendelenburg, U., Ullrich, K., Vogt, W. and Weber, A. – Eds.
Reviews of Physiology, Biochemistry and Pharmacology, vol. 87 953

Ahnefeld, F. W., Bergmann, H., Burri, C., Dick, W., Halmágyi, M., Hossli, G. und Rügheimer, E. – Hrsg.
Klinische Anästhesiologie und Intensivtherapie Band 21. Therapie mit Blutkomponenten 952

Allinger, N. L., Cava, M. P., de Jongh, D. C., Johnson, C. R., Lebel, N. A. und Stevens, C. L.
Organische Chemie. Übersetzt und bearbeitet von H. Gnichtel, F. Klages, G. Koßmehl, H. Kurreck und G. Manecke 952

Batschelet, E.
Einführung in die Mathematik für Biologen. Übersetzt aus dem Englischen 953

Brandenburg, D. and Wollmer, A. – Eds.
Insulin. Chemistry, Structure and Function of Insulin and Related Hormones. Proceedings of the Second International Insulin Symposium Aachen, Germany, September 4–7, 1979 952

Buddecke, E.
Grundriß der Biochemie. Für Studierende der Medizin, Zahnmedizin und Naturwissenschaften 952

Buddrus, J.
Grundlagen der Organischen Chemie 952

Clemmesen, J., Conning, D. M., Henschler D. and Oesch, F. – Eds.
Archives of Toxicology/Supplement 3. Quantitative Aspects of Risk Assessment in Chemical Carcinogenesis. Symposium held in Rome, Italy, April 3–6, 1979 953

Kleine, T. O. – Hrsg.
Neue Labormethoden für die Liquordiagnostik 952

Mross, K. B.
Zellelektrophorese in der Tumordiagnostik: EM-Test. Grundlagen, Material, Methoden, Ergebnisse 953

Noack, S.
Statistische Auswertung von Meß- und Versuchsdaten mit Taschenrechner und Tischcomputer. Anleitungen und Beispiele aus dem Laborbereich 953

Norpoth, K. H. and Garner, R. C. – Eds.
Short-Term Mutagenicity Test. Systems for Detecting Carcinogens 953

Pette, D. – Ed.
Plasticity of Muscle, Proceedings of a Symposium held at the University of Konstanz, September 23–28, 1979 952

Rothmund, M. – Hrsg.
Hyperparathyreoidismus 952

Sauer, H. W.
Entwicklungsbiologie. Ansätze zu einer Synthese. Hochschultext 953

von Voss, H. und Göbel, U. – Hrsg.
Praktische Anwendung der Thrombozytenfunktionsdiagnostik. 2. Symposium „Gerinnungsstörungen im Kindesalter" Schloß Heiligenhofen, Lindlar, 1979 953

GENERAL INDEX/GESAMTREGISTER

Authors' Index/Autorenregister

Aaseth, J. s. Alexander, J. 713
Abel-Telkes, B. s. Endler, A. T. 753
Abuknesha, R. A. 662
Adlercreutz, H. 661, 695
Adolf, P. K. s. Leitz, V. M. 718
Afting, E. G. s. Roethig, H.-J. 756
Agarwal, D. P. s. Meier-Tackmann, Doris 699
Agarwal, D. P. s. Strohmeyer, T. 672
D'Agostino, R. s. Schena, F. P. 17
Agostoni, A. 771
Aiginger, P. 690
Alexander, J. 713
Anderer, F. A. s. Staab, H. J. 175
Arenz, F. s. Lang, C. 551
Arnst, E. s. Koch, C.-D. 423
Arvidsson, E. s. Koch, G. 367
Asbeck, F. s. Schmitz-Huebner, U. 221
Asmuth, Ch. s. Fischer, J. H. 747
Averdunk, R. 710

Baack, G. 726
Bachmann, C. 293
Backs, C. s. Weisweiler, P. 707
Balcke, P. s. Zazgornik, J. 241
Bald, E. 728
Barbe, J. s. Milano, G. 157
Bartmann, P. 1
Bartsch, H.-H. s. Blossey, H.-Ch. 729
Bartsch, W. 730
Bauer, Ch. 670
Bauer, H. J. s. Schipper, H. I. 717
Bauer, K. 677
Bauer, M. s. Wood, W. G. 183, 511
Baumann, K. s. Kochmann, G. 685
Baumgarten, S. s. Schöneshöfer, M. 731
Bayer, P. M. 678
Beck, K. s. Staib, W. 726
Beckermann, W. J. s. Strohmeyer, T. 672
Behbehani, A. s. Langenbeck, U. 755
Belfield, A. s. Pledger, D. R. 701
Belkien, L. s. Jaster, H. J. 742
Bell, P. A. 655
Bencze, K. 13
Benes, P. 117
Bellobuono, A. s. Idéo, G. 237
Berends, G. T. s. Tel, R. M. 595
Beretta, L. s. Agostoni, A. 771
Berg, A. 707
Berg, A. s. Köttgen, E. 752
Bergdahl, B. s. Lindwall, L. 879
Berge, P.-G. s. Wilhelm, A. 271
Bergmeyer, H. U. 521
Bergström, K. 659
Bergström, K. s. Egberg, N. 709
Bernhardt, W. s. Kauerz, U. 745
Bernhardt, W. s. Roethig, H.-J. 756
Bethge, N. 734
Bianchi, M. s. Agostoni, A. 771
Birath, K. 680
Birath, K. s. Skude, G. 680
Biro, G. s. Leicht, E. 739
Bjømaes, H. s. Nordby, H. K. 668
Blaabjerg, O. s. Hyltoft Petersen, P. 744
Bleyl, H. 710
Bleyl, H. s. Hüting, J. 681
Blijenberg, B. G. 27
Blijenberg, B. G. s. Lanser, A. 327
Blombäck, M. s. Wahlberg, R. 744
Blossey, H.-Ch. 729
Blümel, G. s. Zänker, K. S. 728
Blum, H. E. 679
Blum, K. s. Hoffmann, G. E. 129
Bock, K. D. s. Maruhn, D. 686
Boer, P. s. Rijn, H. J. M. van 627
Boer, R. de s. Verheesen, P. E. 351
Bohner, J. s. Stein, W. 677
Bojar, H. 725
Bojar, H. s. Staib, W. 726
Bojar, H. s. Stuhldreier, B. 724
Bombelli, F. s. Agostoni, A. 771
Bomhard, E. s. Maruhn, D. 686
Bonomo, L. s. Schena, F. P. 17
Boos, K. S. s. Schlimme, E. 696
Borner, K. 719
Borst, A. s. Naus, A. J. 621
Borth, R. s. Büttner, J. 535, 855, 861
Bosak, J. 413
Boublil, J. L. s. Milano, G. 157
Boutwell, J. H. s. Büttner, J. 855, 861
Bowyer, R. C. s. Büttner, J. 535, 855, 861
Brandslund, I. s. Klitgaard, N. A. 720
Breidenbach, B. s. Tarkkanen, P. 712
Brendel, W. s. Bartmann, P. 1

Breuer, H. 659, 937
Breuer, H. s. Kaulhausen, H. 137
Breuer, H. s. Nocke-Finck, L. 897
Breuer, H. s. Rao, G. S. 307
Breuer, H. s. Wagener, C. 821
Brombacher, P. J. s. Verheesen, P. N. 351
Broughton, P. M. G. s. Büttner, J. 535, 855, 861
Bruchelt, G. 715
Bruckner, A. 275
Bruno, G. s. Schena, F. P. 17
Büttner, J. 535, 855, 861
Büttner, J. s. Riedel, H. 749
Buss, H. s. Oemar, B. S. 381
Butte, W. 740
Butz, R. s. Wood, W. G. 789

Cambon, P. s. Milano, G. 157
Casaretto, M. s. Wood, W. G. 789
Chmiel, J. 672
Clausen, J. s. Rastogi, S. C. 67
Collet-Cassard, D. s. Holy, H. W. 701
Collombel, Ch. s. Haeckel, R. 445
Colombo, J. P. s. Bachmann, C. 293
Coper, H. s. Honecker, H. 133
Cramarossa, F. s. Schena, F. P. 17
Cremer, P. s. Wieland, H. 749
Cremers, H. M. H. G. s. Verheesen, P. N. 351
Cullmann, W. 671
Cullmann, W. s. Dick, W. 709
Curnow, D. H. s. Porter, C. J. 439
Czernik, A. s. Kleesiek, K. 867

Dabek, J. 665
Dahlmann, N. 682
Damm, D. s. Hajdú, P. 209
Deeg, R. 49
Delogne-Desnoeck, J. s. Jungers, J. 734
Denicke, C. s. Dulce, H. J. 741
Deus, B. 692
Deus, B. s. Berg, A. 707
Deus, B. s. Blum, H. E. 679
Deutsch, E. s. Zazgornik, J. 241
Dick, W. 255, 798
Dick, W. s. Cullmann, W. 671
Dieckvoß, E. s. Harm, K. 694
Diederichs, F. 674
Diederichs, F. s. Friedel, R. 666
Diederichs, F. s. Mühlhaus, K. 674
Diehl, V. s. Oellerich, M. 169
Diel, F. s. Bethge, N. 734
Diener, U. 687
Diener, U. s. Ratge, D. 735
Dietel, M. 739
Dmowska, B. s. Sawicki, Z. 694
Dobler, T. s. Heimsch, E. 775
Dodson, P. M. 751
Dodson, P. s. Stocks, J. 753
Dörken, B. s. Ebert, W. 716
Dörner, K. 713
Dorda, W. s. Müller, M. M. 737
Dorn, G. s. Dietel, M. 739
Dorn-Quint, G. 664

Drosner, M. 750
Drost, H. s. Nagamura, Y. 672
Drygas, J. 751
Dubowitz, V. s. Moss, D. W. 675
Dukwicz, A. s. Sawicke, Z. 694
Dulce, H. J. 741
Durst, R. A. s. Siggaard-Andersen, O. 829
Dyggve, H. V. s. Rastogi, S. C. 67

Eberhard, A. s. Kleesiek, K. 867
Eberl, H. 257
Ebert, W. 716
Egberg, N. 709
Egberg, N. s. Wahlberg, R. 744
Eggstein, M. s. Jakober, B. 708
Eggstein, M. s. Müller, P. H. 708
Ehlers, C. Th. s. Luthe, H. 693
Eijk, H. G. van 593
Endler, A. T. 753
Engelbrecht, S. s. Wenzel, H. R. 699
Engelhardt, P. s. Oellerich, M. 169
Ernst, M. 705
Ernst, M. s. Möller, H. 714
Exley, D. s. Abuknesha, R. A. 662

Fabricius, H. Å. 716
Fabricius, H. Å. s. Köttgen, E. 752
Fähndrich, C. s. Honecker, H. 133
Farrar, G. 376
Fekete, B. s. Tomcsányi, A. 145
Feldt-Rasmussen, U. s. Hyltoft Petersen, P. 744
Fenili, D. s. Zoppi, F. 59
Fenner, A. s. Dulce, H. J. 741
Fenner, A. s. Schöneshöfer, M. 731
Fenninger, H. s. Gibitz, H. J. 721
Fenske, M. 687
Férard, G. 39
Fiederer, B. s. Moeller, H. 726
Fink, E. s. Mann, K. 395
Fink, P. C. 717
Fischer, J. H. 747
Fischer, R. s. Staab, H. J. 175
Fitzgerald, D. E. s. Leitz, V. M. 718
Fogh-Andersen, N. 675
Frey, H. O. 722
Fricke, U. s. Newmann, R. A. 31
Friedel, R. 666
Friedel, R. s. Lindena, J. 649
Fritz, H. s. Jochum, Marianne 671
Fuhs, M. s. Fischer, J. H. 747
Fujita, K. s. Teradaira, R. 698

Gabl, F. s. Bauer, K. 677
Gabl, F. s. Endler, A. T. 753
Gaertner, H. J. 579
Gärtner, R. 571

Galton, D. J. s. Dodson, P. M. 751
Galton, D. s. Stocks, J. 753
Gatschke, W. 403
Gawlik, D. s. Gatschke, W. 403
Geary, T. D. s. Haeckel, R. 445
Geiger, R. s. Mann, K. 395
Geiseler, D. s. Jakober, B. 708
Geseler, D. s. Müller, P. H. 708
Gerbitz, K.-D. 313
Gerli, G. C. s. Agostoni, A. 771
Gerok, W. s. Bauer, Ch. 670
Gersonde, K. s. Sick, H. 689
Geyer, H. s. Baack, G. 726
Gibitz, H. J. 721
Gilboa-Garber, N. 376
Gilch, R. s. Mann, K. 733
Girndt, G. s. Fenske, M. 687
Glöckner, W. M. 377
Goedde, H. W. s. Meier-Tackmann, D. 699
Goedde, H. W. s. Strohmeyer, T. 672
Goedde, H. W. s. Meier-Tack- 754
Göring, W. s. Mann, K. 395
Goerz, G. s. Stuhldreier, B. 724
Götze, M. A. s. Hannak, D. 735
Gollwitzer, Rotraud s. Wood, W. G. 711
Gorecka, A. s. Sawicki, Z. 694
Gorka, G. 697
Graf, H. 686
Graf, H. s. Kovarik, J. 747
Graf, H.-P. s. Trendelenburg, Chr. 732
Greiling, H. s. Gressner, A. M. 279
Gressner, A. M. 111, 279, 684, 797, 921
Gressner, A. M. s. Wallraff, P. 702
Gripenberg, J. s. Jansson, S.-E. 648
Grönroos, P. s. Koivula, T. 686
Groome, N. P. 345
Grospietsch, G. s. Fenske, M. 687
Großmann, M. s. Klingler, W. 700
Grouls, P. s. Gressner, A. M. 684
Gruber, W. s. Markowetz, D. 679
Grüner, J. s. Averdunk, R. 710
Günther, T. s. Ebel, H. 257
Gürtler, L. G. 377
Gundlach, G. 603, 682
Gundlach, G. s. Hüting, J. 681
Gupta, D. s. Moeller, H. 726
Gustafsson, J.-A. 654
Gyr, K. s. Lang, C. 551

Habermann, J. s. Thiel, D. van 807
Hackenberg, K. s. Schön, F. 355
Hadjidimos, A. s. Ratge, D. 735
Haeckel, R. 385, 433, 445, 455, 693
Haeckel, R. s. Heinz, F. 781
Haeckel, R. s. Riedel, H. 749
Hällström, O. s. Koivula, T. 686

Härkönen, M. s. Adlercreutz, H. 661
Hagemeier, E. s. Schlimme, E. 696
Haidl, P. s. Mann, K. 733
Haindl, H. s. Oellerich, M. 718
Hajdú, P. 209
Halket, J. M. 731
Hammerl, B. s. Mann, K. 733
Hamperl, W. s. Jochum, M. 671
Hannak, D. 735
Hansen, I. s. Hørder, M. 679
Hansen, S. I. s. Holm, J. 716
Hansen, S. I. s. Lyngvye, J. 715
Hansert, E. 461
Harada, S. s. Meier-Tackmann, D. 699
Harders, H. D. 743
Harm, K. 694
Harm, K. s. Klapdor, R. 676
Harm, K. s. Rehpenning, W. 743
Harmoinen, A. 702
Harmoinen, A. s. Koivula, T. 686
Harmoinen, A. s. Perko, M. 701
Hartmann, F. s. Butte, W. 740
Hartmann, H. G. s. Maruhn, D. 686
Hartter, P. s. Krupka, U. 723
Hartter, P. s. Martin, W. 723
Hayakawa, S. s. Ikeda, M. 407
Heck, H. s. Hollmann, W. 652
Heckmatt, J. s. Moss, D. W. 675
Hergermann.Nielsen, G. s. Klitgaard, N. A. 720
Hehrmann, R. s. Wood, W. G. 789
Heiden, W. C. H. van 333
Heienbrok, W. s. Klapdor, R. 676
Heikkinen, H. s. Penttilä, I. M. 732
Heimes, J. s. Jäckel, W. 909
Heimsch, E. 775
Heinrigs, S. s. Gressner, A. M. 684
Heinz, F. 781
Helenius, T. s. Liewendahl, K. 737
Helger, R. s. Harders, H. D. 743
Helger, R. s. Vormbrock, R. 698
Hell, B. s. Dulce, H. J. 741
Hellmann, T. s. Mann, K. 733
Henkel, E. 702
Henkel, E. s. Wrabetz-Wölke, A. 703
Henny, J. s. Hubsch, G. 149
Herchenhan, E. s. Blum, H. E. 679
Herndl, R. s. Marschner, I. 105
Herrmann, J. s. Jäckel, W. 909
Hesch, R. D. s. Jüppner, H. 585
Hesch, R. D. s. Wood, W. G. 789
Heul, C. van der s. Eijk, H. G. van 563
Heyes, H. s. Musch, K. 749
Hilz, H. 656
Hinghofer-Szalkay, H. 615
Hinghofer-Szalkay, H. s. Moser, M. 233

Höfer, R. s. Müller, M. M. 737
Hørder, M. 679
Hørder, M. s. Bergmeyer, H. U. 521
Hørder, M. s. Hyltoft Petersen, P. 744
Hoffmann, G. E. 129
Holbolth, N. s. Rosleff, F. 755
Holdsworth, G. s. Dodson, P. M. 751
Holdsworth, G. s. Stocks, J. 753
Hollmann, W. 652
Hollweg, H. G. s. Oemar, B. S. 381
Holm, J. 716
Holm, J. s. Lyngbye, J. 715
Holmberg, H. 681
Holy, H. W. 701
Holz, J. s. Bethge, N. 734
Holzer, H. s. Moser, M. 233
Holzmann, H. s. Benes, P. 117
Homolka, J. 755
Honecker, H. 133
Hoo, J. J. s. Strohmeyer, T. 672
Hoppen, H. O. 729
Horn, K. s. Gärtner, R. 571
Horn, K. s. Wood, W. G. 511
Hornig, D. s. Bruchelt, G. 715
Hotschek, H. s. Bayer, P. M. 678
Houot, O. s. Hubsch, G. 149
Howard Grøn, I. 746
Hubsch, G. 149
Hüllemann, K. s. Weisweiler, P. 707
Hüting, J. 681
Huseby, N.-E. 673
Huttunen, J. K. 652
Hyanek, J. s. Homolka, J. 755
Hyltoft Petersen, P. 744
Hyltoft Petersen, P. s. Hørder, M. 679
Hyltoft Petersen, P. s. Rosleff, F. 755
Hysek, H. s. Zazgornik, J. 241

Ideo, G. 237
Ignatowicz, E. s. Chmiel, J. 672
Ikeda, M. 407
Ikkala, E. s. Rasi, V. 656

Jäckel, W. 909
Jänne, O. s. Jokela, H. 678
Jakober, B. 708
Jakober, B. s. Müller, P. H. 708
Janetschek, P. s. Weisweiler, P. 707
Janssen, E. s. Kania, J. 378
Jansson, S.-E. 648
Jaster, H. J. 742
Jochum, M. 671
Johansson, U. s. Koch, G. 367
Jokela, H. 678
Jokela, H. s. Harmoinen, A. 702
Jokela, H. s. Perko, M. 701
Jonat, W. 727
Jüppner, H. 585
Jüppner, H. s. Wood, W. G. 789
Jungblut, P. W. 653
Jungers, J. 734

Källberg, M. 491
Kärkkäinen, P. 691
Kage, A. 740
Kahn, S. N. 23
Kalbfleisch, U. s. Bald, E. 728
Kalden, J. R. s. Heinz, F. 781
Kamarýt, J. 611
Kania, J. 378
Karduck, D. s. Gilboa-Garger, N. 376
Karges, H. E. s. Pott, G. 893
Karl, H.-J. s. Mann, K. 395, 733
Karonen, S.-L. s. Adlercreutz, H. 661
Kattermann, R. s. Frey, H. O. 722
Kattermann, R. s. Hannak, D. 735
Kattermann, R. s. Sirowej, H. 752
Kauerz, U. 745
Kaul, A. s. Schwörer, I. 163
Kaulhausen, H. 137
Keipert, B. s. Mann, K. 395
Keller, H. s. Haeckel, R. 693
Kenner, Th. s. Moser, M. 233
Kertész, L. s. Tomcsányi, A. 145
Kessler, A.-Ch. s. Schlebusch, H. 885
Keul, J. s. Berg, A. 707
Kewenig, M. s. Gärtner, R. 571
Khan, R. A. s. Kahn, S. N. 23
Kiss, P. s. Sirowej, H. 752
Klapdor, R. 676
Kleesiek, K. 867
Klein, P. J. 378
Klein, P. J. s. Farrar, G. 376
Klein, P. J. s. Kania, J. 378
Klein, P. J. s. Müller, W. 380
Klein, P. J. s. Vierbuchen, M. 384
Klein, P. J. s. Newman, R. A. 31
Kleine, T. O. 7, 245
Klingler, W. 700
Klitgaard, N. A. 720
Klötzl, G. 725
Klose, B.-J. s. Schramel, P. 591
Klosse, J. A. s. Rijn, H. J. M. van 627
Klumpp, T. s. Férard, G. 39
Knedel, M. s. Haeckel, R. 693
Knoll, E. 53
Knoll, E. s. Bosak, J. 413
Knoll, E. s. Diener, U. 687
Knoll, E. s. Ratge, D. 835
Knoll, E. s. Wisser, H. 297
Knoth, E. s. Bayer, P. M. 678
Koch, C.-D. 423
Koch, G. 367
Kochmann, G. 685
Kochmann, R. s. Kochmann, G. 685
Koebberling, J. s. Blossey, H.-Ch. 729
Koester-Eiserfunke, W. s. Gressner, A. M. 279, 684
Köttgen, E. 379, 752
Köttgen, E. s. Bauer, Ch. 670
Köttgen, E. s. Fabricius, H. Å. 716
Koivula, T. 686
Koivula, T. s. Harmoinen, A. 702
Koivula, T. s. Jokela, H. 678
Kolb, H. s. Nagamura, Y. 672
Kopsa, H. s. Zazgornik, J. 241
Kornmüller, K. 711
Korte, T. 721
Kothaus, G. s. Nagamura, Y. 672
Kovarik, J. 747
Kraehenmann, J. A. s. Lang, C. 551
Kraemer, W. s. Deeg, R. 49
Kraft, D. s. Gatschke, W. 403
Krais, J. s. Stein, W. 677
Krauth-Siegel, L. 724
Krawczyński, J. s. Sawicki, Z. 694
Krieg, M. s. Klötzl, G. 725
Kroos, M. J. s. Eijk, H. G. van 563
Krupka, U. 723
Kruse-Jarres, J. D. s. Trendelenburg, Chr. 732
Kühn, H. s. Luthe, H. 693
Külpmann, W. R. 227, 339, 720
Kuhn, W. s. Fenske, M. 687
Kump. B. s. Bojar, H. 725
Kuoppasalmi, K. 653
Kuppens, P. S. s. Naus, A. J. 621
Kuss, H. J. s. Gaertner, H. J. 579
Kuzmits, R. 745
Kuzmits, R. s. Aiginger, P. 690

Lafosse, M. s. Kaulhausen, H. 137
Lalanne, C. M. s. Milano, G. 157
Lalla, M. 688
Lang, C. 551
Lang, H. s. Aiginger, P. 690
Lang, W. s. Wolf, H. U. 689
Lange, H. s. Luthe, H. 693
Langenbeck, U. 755
Langer, B. s. Diener, U. 687
Lanser, A. 327
Lantta, M. s. Stenman, U.-H. 727
Latipää, P. s. Jokela, H. 678
Lauritzen, Ch. s. Müller-Oesterle, R. 748
Lauritzen, Ch. s. Wolf, A. S. 747
Lehtinen, Inkeri s. Puukka, M. 692
Leicht, E. 739
Leijnse, B. s. Blijenberg, B. G. 27
Leijnse, B. s. Lanser, A. 327
Leitz, V. M. 718
Lemm, U. s. Wenzel, M. 684
Leskinen, E. s. Holmberg, H. 681
Leur, E. Van de s. Gressner, A. M. 279
Lewis, S. M. 649
Liesen, H. s. Hollmann, W. 652
Liewendahl, K. 737
Linde, H. s. Deus, B. 692
Lindena, J. 649
Lindholm, A.-M. s. Adlercreutz, H. 661
Lindwall, L. 879
Linkesch, W. s. Kovarik, J. 747
Lipp, W. s. Mann, K. 395
Lode, H. s. Borner, K. 719
Lorentz, K. 379, 682
Lorke, D. s. Maruhn, D. 686
Lück, H. J. s. Hoppen, H. O. 729
Lüllig, H. s. Ebert, W. 716
Luthe, H. 693
Luthe, H. s. Langenbeck, U. 755
Luttermann, E. s. Gundlach, G. 682
Lyngbye, J. 715
Lyngbye, J. s. Holm, J. 716

Maas, A. H. J. s. Siggaard-Andersen, O. 829
Maass, H. s. Jonat, W. 727
Mach, M. s. Tomaszewski, J. J. 750
Mader, A. s. Hollmann, W. 652
Magdziarz, A. s. Palynyczko, Z. 676
Magid, E. 669
Magnusson, C. G. s. Holy, H. W. 701
Mann, K. 395, 733
Manno, C. s. Schena, F. P. 17
Marcovina, S. s. Zoppi, F. 59
Markowetz, D. 679
Marschner, I. 105, 660
Marschner, I. s. Thiel, D. van 807
Marschner, I. s. Wood, W. G. 183, 511, 789
Martin, W. 723
Maruhn, D. 686
Maruhn, D. s. Paar, D. 557
Maruta, K. s. Teradaira, R. 698
Masson, P. S. s. Holy, H. W. 701
Matýsek, P. s. Kamarýt, J. 611
McGeeney, F. 673
Meier-Tackmann, D. 699
Melchior, J. C. s. Rastogi, S. C. 67
Merk, H. s. Stuhldreier, B. 724
Merten, B. s. Kleine, T. O. 245
Métais, P. s. Férard, G. 39
Mette, H. s. Klingler, W. 700
Mettinger, K. L. s. Wahlberg, R. 744
Metzger, G. s. Férard, G. 39
Meulen, N. van der s. Bald, E. 728
Meyering, M. s. Pott, G. 893
Michaelis, H. C. s. Thomas, L. 697
Milano, G. 157
Mitchell, F. L. s. Haeckel, R. 445
Miyato, M. s. Fischer, J. H. 747
Moeller, H. 726
Möller, H. 714
Mohr, H. s. Jüppner, H. 585
Molin, L. s. Lindwall, L. 879
Molnar, I. s. Schöneshöfer, M. 731
Mordasini, R. C. 917
Morsches, B. s. Benes, P. 117
Moser, M. 233
Moss, D. W. 675
Mühlhaus, K. 674
Mühlhaus, K. s. Diederichs, F. 674
Mühlhaus, K. s. Friedel, R. 666

Mühlhausen, B. s. Gundlach, G. 603
Müller, M. s. Stein, W. 677
Müller, M. J. 738
Müller, M. M. 737
Müller, M. M. s. Aiginger, P. 690
Müller, M. M. s. Graf, H. 686
Müller, M. M. s. Kuzmits, R. 745
Müller, N. s. Dick, W. 709
Müller, P. H. 708
Müller, P. H. s. Jakober, B. 708
Müller, W. 380
Mössner, W. s. Köttgen, E. 379
Müller, W. E. G. 380
Müller, W. E. G. s. Vaith, P. 383
Müller-Matthesius, R. 501
Müller-Oesterle, R. 748
Müller-Plathe, O. 690
Munz, E. s. Markowetz, D. 679
Munz, E. s. Schlebusch, H. 885
Musch, K. 749
Musch, K. s. Müller-Oesterle, R. 748
Musch, K. s. Wolf, A. S. 747
Myllylä, G. s. Rasi, V. 656

Nachbar, J. s. Schmitz-Huebner, U. 221
Nadeau, R. G. s. Haeckel, R. 445
Näveri, H. s. Kuoppasalmi, 653
Nagamura, Y. 672
Nagatsu, T. s. Teradaira, R. 698
Nage, H.-H. s. Harm, K. 694
Nagel, M. 431
Nakamura, M. s. Teradeira, R. 698
Nanba, N. s. Ikeda, M. 407
Naus, A. J. 621
Newmann, R. A. 31
Nicolai, H. von 381

Nocke, Finck, L. 897
Noort, W. L. van s. Eijk, H. G. van 563
Nordby, H. K. 668

Oellerich, M. 169, 197, 718
Oellerich, M. s. Külpmann, W. R. 720
Oemar, B. S. 381
Oettling, G. s. Moeller, H. 726
Ohmori, S. s. Ikeda, M. 407
Okuda, K. 947
Okuda, K. s. Haecke., R. 445
Oldendörp, J. 901
Olek, K. 567
Olesen, E. s. Winkel, P. 648
Opper, C. s. Müller-Matthesius, R. 501
Osburg, K. s. Drygas, J. 751
Oster, O. 714
Overkamp. D. s. Müller, P. H. 708

Paar, D. 557
Paar, D. s. Maruhn, 686
Paar, D. s. Schön, F. 355
Pai, E. F. s. Krauth-Siegel, L. 724
Palynyczko, Z. 676
Pape, W. s. Kochmann, G. 685
Parmar, C. s. Moss, D. W. 675
Patscheke, H. 708
Pabelka, R. s. Kovarik, J. 747
Penttilä, A. s. Korte, T. 721
Penttilä, I. s. Puhakainen, E. 684
Penttilä, I. M. 732
Penttilä, I. M. s. Kärkkäinen, P. 691
Peracino, A. s. Zoppi, F. 59
Perko, M. 701
Perko, M. s. Harmoinen, A. 702
Persijn, J.-P. s. Heiden, W. C. H. van 333
Petersen, E. s. Wrabetz-Wölke, A. 703
Petzinna, D. s. Bojar, H. 725
Pianezzi, A. s. Scholer, A. 363
Pikkarainen, J. s. Korte, T. 721
Pilaski, J. s. Staib, W. 726
Pils, P. s. Zazgornik, J. 241
Pilz, R. s. Heinz, F. 781
Pledger, D. R. 701
Porter, C. J. 439
Pott, G. 893
Prokscha, G. W. s. Zänker, K. S. 728
Puhakainen, E. 684
Puukka, M. 497, 692
Puukka, M. s. Puukka, R. 706
Puukka, R. 706
Puukka, R. s. Puukka, M. 497, 692

Quabbe, H. J. s. Bethge, N. 734

Räsänen, V. 700
Räsänen, V. s. Lalla, M. 688
Ragagnin, M. s. Férard, G. 39
Rao, G. S. 307
Rao, V. H. s. Sambasiva Rao, J. 287
Rasi, V. 656
Rasmussen, J. s. Rosleff, F. 755
Rastetter, J. 647
Rastogi, S. C. 67
Ratge, D. 735
Ratge, D. s. Bosak, J. 413
Ratge, D. s. Diener, U. 687
Rausch-Stroomann, J.-G. s. Ernst, M. 705
Rautenstrauch, H. s. Diener, U. 687
Rautenstrauch, H. s. Knoll, E. 53
Reckel, S. s. Heinz, F. 781
Refsum, H. E. 651
Rehpenning, W. 743
Rehpenning, W. s. Kauerz, W. 745
Reiber, H. 123, 696
Reiber, H. O. s. Schipper, H. I. 717
Reimers, D. s. Nocke-Finck, L. 897
Reinauer, H. s. Oldendörp, J. 901
Reinhard, U. s. Jakober, B. 708
Reinhard, U. s. Müller, P. H. 708
Reinwein, D. s. Schön, F. 355
Renee, N. s. Milano, G. 157
Reunanen, M. s. Puukka, M. 497
Reuschel-Janetschek, E. s. Richter, W. 736
Reutter, W. s. Bauer, Ch. 670
Richter, W. 736
Richter, W. s. Schwandt, P. 736
Rickenbach, R. van s. Scholer, A. 363
Riedel, H. 749
Riesen, W. E. s. Mordasini, R. C. 917
Rijn, H. J. M. van 627
Ringwald, J. s. Berg, A. 707
Robyn, C. s. Jungers, J. 734
Röhle, G. s. Breuer, H. 659
Röhr, A. s. Schlaeger, R. 695
Rösick, N. s. Bethge, N. 734
Röthig, H.-J. 756
Röthig, H. J. s. Weisner, B. 746
Rohner, H. G. s. Kaulhausen, H. 137
Róka, L. s. Bleyl, H. 710
Rommel, K. s. Koch, C.-D. 423
Rommelspacher, H. s. Honecker, H. 133
Rosleff, F. 755
Rüdiger, H. 382
Ruppenthal, E. s. Schmidt, R. 748
Ryhänen, R. s. Puhakainen, E. 684

Sabatke, L. s. Bencze, K. 13
Salaspuro, M. s. Liewendahl, K. 737
Sambasiva, Rao, J. 287
Sawicki, Z. 694
Schaast, M. s. Oellerich, M. 169
Schena, F. P. 17
Schieb, E. s. Vaith, P. 383
Schipper, H. I. 717
Schirmer, R. H. s. Krauth-Siegel, Luise 724
Schlaeger, R. 695
Schlebusch, H. 885
Schlimme, E. 696
Schmid, G. s. Wisser, H. 297
Schmid, K. s. Müller, P. H. 708
Schmidt, E. 668
Schmidt, K. s. Bruchelt, G. 715
Schmidt, P. s. Zazgornik, J. 241
Schmidt, R. 748
Schmitt, S. s. Köttgen, E. 379
Schmitz-Huebner, U. 221
Schmülling, R. M. s. Jakober, B. 708
Schmülling, R.-M. s. Müller, P. H. 708
Schneider, M. s. Milano, G. 157
Schneider, W. 691
Schön, F. 355
Schöneshöfer, M. 731
Schöneshöfer, M. s. Dulce, H. J. 741
Schöneshöfer, M. s. Jaster, H. J. 742
Schöneshöfer, M. s. Kage, A. 740
Scholer, A. 363
Schottelius, J. 383
Schramel, P. 591
Schramm, W. s. Wood, W. G. 711
Schriewer, H. s. Wihlem, A. 271
Schröder, H. s. Hilz, H. 655
Schubert, G. s. Frey, H. O. 722
Schümann, H.-J. s. Nagel, M. 431
Schultze, K.-D. s. Fink, P. C. 717
Schulz, G. E. s. Krauth-Siegel, L. 724
Schumacher, W. s. Müller-Plathe, O. 690
Schwandt, P. 736
Schwandt, P. s. Drosner, M. 750
Schwandt, P. s. Richter, W. 736
Schwandt, P. s. Weisweiler, P. 707
Schwarz, S. 215
Schweiger, O. s. Tomcsányi, A. 145
Schwörer, I. 163
Scriba, P. C. s. Gärtner, R. 571
Scriba, P. C. s. Marschner, I. 105, 660
Scriba, P. C. s. Thiel, D. van 807
Scriba, P. C. s. Wood, W. G. 183, 511
Seeger, H. T. 706
Seidel, D. s. Luthe, H. 693
Seidel, D. s. Walli, A. 754
Seidel, D. s. Wieland, H. 749
Seitz, H. J. s. Müller, M. J. 738
Sewry, C. s. Moss, D. W. 675
Shinpo, K. s. Teradaira, R. 698
Shortman, R. C. s. Kahn, S. N. 23
Sick, H. 689
Sieber, A. s. Pott, G. 893
Siedek, M. s. Kaulhausen, H. 137
Siggaard-Andersen, O. 929
Singh, S. s. Willers, I. 754
Sirowej, H. 752
Sirowej, H. s. Frey, H. O. 722
Skinningsrud, A. 669
Skude, G. 680
Skude, G. s. Birath, K. 680
Slik, W. van der s. Heiden, W. C. H. van 333
Sloman, J. C. s. Bell, P. A. 655
Sluijs Veer, G. van der 305
Sommer, B. s. Howard Grøn, I. 746
Sorger, M. s. Schlebusch, H. 885
Sorto, A. s. Jokela, H. 678
Souverijn, J. H. M. s. Heiden, W. C. H. van 333
Staab, H. J. 175
Stadler, H. s. Sirowej, H. 752
Stahn, R. s. Fabricius, H. Å. 716
Staib, W. 726
Staib, W. s. Bojar, H. 725
Staib, W. s. Stuhldreier, B. 724
Staiger, Ch. 817
Stamm, D. s. Hansert, E. 461

Steffens, E. s. Gorka, G. 697
Stein, W. 677
Stenflo, J. 657
Stenman, S. s. Stenman, U.-H. 727
Stenman, U.-H. 727
Stinshoff, K. s. Gorka, G. 697
Stocks, J. 753
Stocks, J. s. Dodson, P. M. 751
Strömber, P. s. Pledger, D. R. 701
Strømme, J. H. s. Nordby, H. K. 668
Strohmeyer, T. 672
Struck, H. s. Unger, Th. 631
Stuhldreier, B. 724
Stummvoll, H. K. s. Graf, H. 686
Stumpf, E. s. Staab, H. J. 175
Suhartono, H. s. Gundlach, G. 682
Sutor, D. J. 193
Svanberg, T. s. Puukka, M. 692

Tanner, P. s. Liewendahl, K. 737
Tarkkanen, P. 712
Tel, R. M. 595
Tempini, S. s. Idéo, G. 237
Teradaira, R. 698
Theodorsen, L. s. Skinningsrud, A. 669
Thiel, D. van 807
Thiel, D. van s. Wood, W. G. 511
Thom, R. 647
Thomas, J. s. Tarkkanen, P. 712
Thomas, L. 697
Thomas, M. s. Sirowej, H. 752
Thompson, E. J. s. Kahn, S. N. 23
Thomsen, P. D. s. Bethge, N. 734
Thygesen, K. s. Hørder, M. 679
Thygesen, K. s. Hyltoft Petersen, P. 744
Tietz, N. W. 763
Tobiasson, P. s. Källberg, M. 491
Tomaszewski, J. J. 750
Tomcsányi, A. 145
Trautschold, I. s. Diederichs, F. 674
Trautschold, I. s. Mühlhaus, K. 674
Trendelenburg, Chr. 732
Trettin, H. s. Leitz, V. M. 718
Trnka, V. s. Homolka, J. 755
Trombik, E. s. Müller, M. M. 737
Tschesche, H. s. Wenzel, H. R. 699

Ueckert, B. s. Trendelenburg, Chr. 732
Untucht-Grau, R. s. Krauth-Siegel, L. 724
Uhlenbruck, G. 373
Uhlenbruck, G. s. Farrar, G. 376
Uhlenbruck, G. s. Gilboa-Garber, N. 376
Uhlenbruck, G. s. Kania, J. 378
Uhlenbruck, G. s. Klein, P. J. 378
Uhlenbruck, G. s. Müller, W. 380
Uhlenbruck, G. s. Müller, W. E. G. 380
Uhlenbruck, G. s. Newman, R. A. 31
Uhlenbruck, G. s. Vaith, P. 383
Uhlenbruck, G. s. Vierbuchen, M. 384
Uhlhaas, S. s. Olek, K. 567
Uihlein, M. s. Hajdú, P. 209
Uldall, A. 303
Unger, Th. 631
Urdal, P. s. Nordby, H. K. 668

Vaith, P. 383
Vaith, P. s. Müller, W. E. G. 380
Verheesen, P. E. 351
Vierbuchen, M. 384
Vierbuchen, M. s. Farrar, G. 376
Vierbuchen, M. s. Kania, J. 378
Vierbuchen, M. s. Klein, P. J. 378
Vierbuchen, M. s. Müller, W. 380
Vignali, M. s. Agostoni, A. 771
Vihko, P. 682
Vihko, R. 654
Virkola, P. s. Adlercreutz, H. 695
Voigt, K. D. s. Harm, K. 694
Voigt, K. D. s. Klötzl, G. 725
Voigt, K. D. s. Rehpenning, W. 743
Vonderschmitt, D. J. s. Scholer, A. 363
Vormbrock, R. 698
Voss, B. s. Pott, G. 893
Vossen, J. G. H. M. 712
Vries, A. L. De s. Newman, R. A. 31
Vries, J. de s. Staiger, Ch. 817

Wachter, C. s. Wood, W. G. 511, 789
Wagener, C. 821
Wahlberg, R. 744
Wahlstedt, Virve s. Holmberg, Helena 681
Walb, D. s. Thomas, L. 697
Wallenburg, H. C. S. s. Lanser, A. 327
Walli, A. 754
Wallraff, P. 702
Wallraff, P. s. Gressner, A. M. 797
Walter, E. s. Staiger, Ch. 817
Wardenbach, P. s. Olek, K. 567
Weber, B. s. Dulce, H. J. 741
Weber, B. s. Jaster, H. J. 742
Weber, B. s. Kage, A. 740
Weber, W. s. Hilz, H. 655
Wegener, M. s. Harm, K. 694
Wehner, B. s. Klein, P. J. 378
Weinstock, N. 712
Weise, M. s. Schlimme, E. 696
Weisner, B. 746
Weisner, B. s. Kauerz, U. 745
Weiss, L. s. Hoffmann, G. E. 129
Weissel, M. s. Müller, M. M. 737
Weisweiler, P. 707
Weisweiler, P. s. Drosner, M. 750
Wenzel, H. R. 699
Wenzel, M. 684
Wenzel, M. s. Fischer, J. H. 747
Werle, S. s. Frey, H. O. &22
Whitaker, K. B. s. Moss, D. W. 675
Whitehead, T. P. 453
Wiatr, G. s. Gaertner, H. J. 579
Wider, G. s. Bayer, P. M. 678
Wiechel, B. s. Wahlberg, R. 744
Wieland, H. 749
Wieland, H. s. Luthe, H. 693
Wiest, W. s. Schmidt, R. 748
Wilhelm, A. 271
Wilkie, L. I. s. Sutor, D. J. 193
Willers, I. 754
Wiman, B. 657
Winckelmann, M. s. Thomas, L. 697
Winkel, P. 648
Wisser, H. 297
Wisser, H. s. Bosak, J. 413
Wisser, H. s. Diener, U. 687
Wisser, H. s. Knoll, E. 53
Wisser, H. s. Ratge, D. 735
Witkowski, J. s. Moss, D. W. 675
Witt, I. 658
Witte, G. s. Henkel, E. 702
Witte, J. s. Jochum, M. 671
Wolf, A. s. Schramel, P. 591
Wolf, A. S. 747
Wolf, A. S. s. Müller-Oesterle, R. 748
Wolf, H. U. 689
Wolfrum, R. s. Halket, J. M. 731
Woloszczuk, W. s. Kovarik, J. 747
Wood, W. G. 183, 511, 711, 789
Wood, W. G. s. Marschner, I. 660
Wood, W. G. s. Thiel, D. van 807
Wrabetz-Wölke, A. 703
Wunderlich, I. s. Dulce, H. J. 741
Wurm, H. s. Hinghofer-Szalkay, H. 615

Zänker, K. S. 728
Zahn, H. s. Wood, W. G. 789
Zander, R. s. Wolf, H. U. 689
Zaugg, H. 545
Zazgornik, J. 241
Ziegenhorn, J. s. Deeg, R. 49
Zilliken, F. s. Nicolai, H. von 381
Zoppi, F. 59
Zwez, W. s. Schlebusch, H. 885
Zyman, H. s. Endler, A. T. 753

Subject Index

absorption spectrum
–, clobazepam 209
ABTS
–, peroxidase assay 345
accuracy 69, 78
N-acetyl-β-D-glucosaminidase
–, urine 53, 687
––, determination 53
–––, value, diagnostic 53
––, under amikacin therapy 687
––, under cisplatin therapy 687
N-acetyl neuraminic acid
–, inhibitor 708
––, thromboxane synthesis 708
acetyl salicylic acid
–, bioavailability 720
N-acetyl transferase
–, synchronization of cell function 763
acute myeloid leukaemia
–, stem cell population monitoring 648
adenocarcinoma
–, gastrointestinal tract 175
––, carcinoembryonic antigen 175
adenosine
–, nucleotides 712
––, platelets 712
–––, determination 712
––––, by bioluminescence 712
adenylate cyclase
–, liver, foetal 748
––, and chorionic gonadotropin 748
adrenal steroid hormones
–, plasma 740
––, circadian variation 740
––, episodic variation 740
adsorption
–, on surfaces 585
––, parathyrin 585
affective diseases
–, function test 867
––, neuroendocine 867
affinity chromatography
–, oligosaccharides 381
–, T-cell receptors 1
age
–, plausibility intervals 694
age dependency
–, lactate dehydrogenase 305
–, uric acid 745
––, serum 745
alanine aminopeptidase
–, urine 687
––, under amikacin therapy 687
––, under cisplatin therapy 687
alanine aminotransferase
–, IFCC method 521
–, stability 521
albumin
–, cerebrospinal fluid 7, 245

albumin
–, serum 96
––, standard 96
alcohol
–, blood 721
––, method comparison 721
–, and drugs 721
alcohol dehydrogenase
–, blood 699
–, fibroblasts 699
–, hair roots 699
–, organs 699
aldehyde dehydrogenase
–, blood 699
–, fibroblasts 699
–, hair roots 699
–, organs 699
aldosterone
–, urine 742
––, high performance liquid chromatography 742
––, radioimmunoassay 742
aluminium
–, bone biopsy 403
––, neutron activation analysis 403
–, serum 591
––, determination 591
––, reference values 591
amikacin
–, therapy 687
––, N-acetal-β-D-glucosaminidase 687
–––, urine 687
––, alanine aminopeptidase 687
–––, urine 687
amino acids
–, physical exercise 651
2-amino-2-methyl-1-propanol
–, buffer substance 698
––, phosphatase, alkaline 698
–––, stabilization 698
aminopeptidases
–, placenta 631
β-aminopropionitrile
–, monoamine oxidase 921
––, inhibitor 921
ammonia
–, physical exercise 651
amniotic fluid
–, fibronectin 797
–, β-glucuronidase 611
––, and sex of the child 611
–, proteases 749
––, lung maturity 749
amylase
–, isoenzymes 669, 673, 680, 681
––, clinical value 669
––, determination 673, 680, 681
analysers
–, specifications 947
––, guidelines 947
analysis
–, clinical chemical 433
––, comparison 433
–––, statistical problems 433
androgens
–, thin layer chromatography 117
5-androsten-3β, 17β-diol
–, plasma 117
––, determination 117
antibiotics
–, and hormone concentrations 897
––, blood 897
antiepileptics
–, serum 720
––, enzyme immunoassay 720
––, gas chromatography 720
anti-Factor VIII clotting activity
–, radioimmunoassay 711
antigen
–, carcinoembryonic 175
antigen specificity
–, T-cell receptors 1
α_2-antiplasmin
–, plasma 221
––, determination 221
antipyrine®
–, plasma 817
––, gas chromatography 817
anti *Thomsen-Friedenreich* agglutinins
–, isolation 378
antithrombin III
–, plasma 221
––, determination 221
α_1-antitrypsin
–, in dialyzed patients 241
–, after kidney transplantation 241
–, phenotyping 13
––, clinical relevance 13
apallic syndrome
–, catecholamines 735
––, circadian rhythm 735
apamin 723
apolipoprotein A-I
–, electroimmunoassay 917
–, radioimmunoassay 917
apolipoprotein A-II
–, electroimmunoassay 917
–, radioimmunoassay 917
apolipoprotein C
–, and bezafibrate 750
–, and dialysis 751
apolipoprotein C-III-2 753
apolipoproteins 707
arsenic
–, determination 714
––, atomic absorption spectrometry 714
arterial walls
–, and plasmatic coagulation system 545
–, thromboplastic activity 545
arylesterase
–, determination 683
–, inhibition 683
––, by lanthanum 683
aryl-4-hydroxylase 775
ascorbate
–, interference 363
––, uric acid assay 363
ascorbate oxidase 363
ascorbic acid
–, urine 715
––, determination 715
–––, isotachophoresis 715
asialoglycoproteins
–, fate 31
––, in mouse circulation 31
assigned values
–, control specimens 461
––, determination 461
–––, matrix effects 461
–––, method dependence 461
atomic absorption spectrometry
–, arsenic 714
–, copper 712
–, mercury 714
–, zinc 712
ATP
–, determination 686
––, bioluminescence assay 686
–––, urine analysis 686
–, leukaemia cells 690
––, and cytotoxic substances 690
AutoAnalyzer II
–, glucose determination 255
––, blood 255
automation
–, definition 455
automatization
–, definition 455
–, perspectives 455

bacteriuria
–, screening 712
––, bioluminescence 712
bee venom peptides
–, and granulocyte migration 723
N-benzoyl-*L*-tyrosyl-*p*-aminobenzoic acid
–, pancreatic function test 551
–, toxicity 551
benzopyrene-3-monooxygenase 775
betamethasone
–, and hormones in pregnancy 748
bezafibrate
–, hyperlipoproteinaemia IV 750
Bhattacharya plot 621
bile
–, copper excretion 713
––, and glutathione depletion 713
––, and paracetamol 713
––, and penicillamine 713
–, palmitic acid 193
––, free 193
–, zinc excretion 713
––, and glutathione depletion 713
––, and paracetamol 713
––, and penicillamine 713
bile acids
–, glycine conjugation 407
––, colorimetry 407
bilirubin
–, cerebrospinal fluid 7
–, total 27
––, serum 27
–––, neonates 27
––––, survey report 27
bioavailability
–, acetylsalicylic acid 720
bioluminescence
–, adenosine nucleotides 712
–, ATP assay 686
–, bacteriuria screening 712
biotransformation
–, enzymes 775
––, quality control 775
biuret reagent 697
bleomycin
–, leukaemia treatment 690
––, ATP 690
blood see also cord blood erythrocytes, plasma, serum
–, alcohol 721
––, method comparison 271
–, alcohol dehydrogenase 699
–, aldehyde dehydrogenase 699
blood see also cord blood erythrocytes, plasma, serum
–, coagulation 744
––, reference material 744
–, ethanol 722
––, microdetermination 722
–, glucose 129, 149, 255, 275, 885
––, determination 129, 885
–––, and deproteinization 129
–, men 897
––, cortisol 897
–––, after rifampicin 897
–––, after streptomycin 897
––, testosterone 897
–––, after rifampicin 897
–––, after streptomycin 897
–, occult 695
––, faeces 695
–, phenylpyruvic acid 755
–, thyrotropin 807
––, screening 807
blood/brain barrier
–, dysfunction 717
––, IgG 717
blood gases 829
blood group 4, 383
bone
–, biopsy 403
––, aluminium 403
––, phosphorus 403
brain
–, acute damage 668
––, creatine kinase-BB 668
–, magnesium 257
breast cancer
–, catechol-O-methyltransferase 763
–, receptor assay 727, 728, 729
bupivacaine
–, plasma 351
––, gas chromatography 351

C-peptide
–, plasma 313
–, urine 313
C-reactive protein
–, determination 710
C1 esterase
–, inhibitor 17
––, assay 17
–––, hereditary angioneurotic edema 17
––, inhibition mechanism 17
–, *Michaelis* constant
Ca^{2+}-paradox
–, enzyme release 674
––, by rat heart 674
calcitonin
–, assay 664
–, serum 747
––, neonates 747
––, pregnancy 747
calibration 855
cAMP
–, plasma 909
––, after glucagon 909
–, radioimmunoassay 732
––, inhibition by EDTA 732
–, receptors 656
––, tissue 656
–––, malignant 656
cancer
–, polyamines 698
––, urine 698
cancer therapy
–, monitoring 763

carbamazepin
–, serum 227
––, determination 227
carbohydrates
–, content 31
––, glycoproteins 31
carbon dioxide
–, partial pressure 829
carbon tetrachloride
–, and protein phosphorylation 111
carcinoembryonic antigen 821
–, adenocarcinoma 175
––, gastrointestinal tract 175
–, immune complexes 175
carcinogenesis
–, steroid epoxides 937
catabolism
–, enzymes 679
catalase
–, erythrocytes 771
––, cord blood 771
––, maternal 771
catecholamines
–, circadian rhythm 735
––, apallic syndrome 735
–, conjugated 431
––, plasma 431
–––, determination 431
–, methylation 413
––, enzymatic 413
–, plasma 413
–, response 367
––, to exercise 367
–, urine 567
catechol-O-methyltransferase
–, breast tissue 763
–, radioenzymatic assay 672
––, hair root cells 672
CEA 175, 821
cefotaxime
–, serum 719
––, determination 719
–––, by HPLC 719
–, urine 719
––, determination 719
–––, by HPLC 719
cerebrospinal fluid
–, albumin 245
–, circulation 746
––, prealbumin as indicator 746
–, cisternal 745
––, glucose 745
–, diagnosis 7
––, with rapid tests 7
–, fibronectin 797
–, glucose 129, 275
––, determination 129
–––, and deproteinization 129
–, IgA 245
–, IgG 245
–, IgM 245
–, lactate 746
––, meningitis purulenta 746
–, lumbar 745
––, glucose 745
–, α_2-macroglobulin 671
–, peptide hormones 731
––, separation 731
–, prealbumin 245
–, production 746
––, prealbumin as indicator 746
–, protein pattern 23
––, polyacrylamide gel electrophoresis 23
–––, sample preparation 23
cerebrospinal fluid
–, proteins 7, 123
––, adsorption 7
–––, to reaction vessels 7
––, nephelometry 123
–, ventricular 745
––, glucose 745
circadian rhythm see also variation
–, catecholamines 735
––, apallic syndrome 735
cisplatin
–, leukemia treatment 690
––, ATP 690
–, therapy 687
––, N-acetyl-β-D-glucosaminidase 687
–––, urine 687
––, alanine aminopeptidase 687
–––, urine 687
Chagas disease
–, trypanosomes 383
––, characterization 383
–––, by lectins 383
chenodeoxycholic acid
–, serum 491
––, radioimmunoassay 491
children
–, reference values 713
––, copper 713
–––, serum 713
–––, urine 713
––, zinc 713
–––, serum 713
–––, urine 713
chloride
–, urine 703
cholestasis
–, extrahepatic 111
––, and protein phosphorylation 111
cholesterol see also HDL, LDL
–, method comparison 595, 752
cholesterolesterase
–, specificity 595
cholesteryl esters
–, hydrolysis 595
cholic acid
–, serum 491
––, radioimmunoassay 491
chorionic gonadotropin
–, adenylate cyclase 747
––, liver, foetal 747
–, determination 733
β-chorionic gonadotropin 821
chromate
–, tubular damage 686
chromatography see high performance liquid, ion exchange, thin layer
chromogenic substrates 631
chromogenic substrate S-2444 557
Chromozyms 709
Chromozym TRY 671
chymotrypsin
–, faeces 695
––, determination 695
clearance
–, creatinine 423
––, variation 423
–, urea 423
––, variation 423
clinical chemistry
–, perspectives 453, 455
––, automatization 455
–, postgraduate course 439
clobazepam
–, serum 209
––, determination 209
–, urine 209
––, determination 209
clotting tests see also coagulation
–, and glycerophosphatides 711
coagulation see also clotting
–, determination 659, 709, 710
––, chromogenic substrate 659
––, non-carboxylated factors 710
–, reference material 744
coagulation system
–, plasmatic 545
––, and thromboplastic activity 545
–––, of arterial walls 545
cold agglutinins
–, monoclonal 716
––, for glycoconjugate-associated antigens 716
–––, neoplastic cells 716
cold insoluble globulin
–, laser nephelometry 702
collagen
–, metabolism 287
––, protein malnutrition 287
colloid osmotic pressure
–, plasma 233
––, determination 233
competitive protein binding assay
–, progesterone 355
complement 17
computer processing
–, radioimmunoassay data 660
computer programme
–, HP 97 215
––, radioimmunoassay 215
confidence intervals
–, radioimmunoassay 879
consensus value 461
control materials 855
control specimens
–, assigned values 461
––, determination 461
copper
–, excretion 713
––, in rat bile 713
–––, and glutathione depletion 713
–––, and paracetamol 713
–––, and penicillamine 713
–, serum 712
––, determination 712
–––, atomic absorption spectrometry 712
–––, reference values 713
––––, children 713
–, urine 713
––, reference values 713
–––, children 713
cord blood see also erythrocytes
–, creatine kinase 675
––, *Duchenne* muscular dystrophy 675
corticosteroid
–, binding globulin 137
––, binding capacity 137
–––, in liver cirrhosis 137
corticotropin
–, plasma 740
––, circadian variation 740
––, episodic variation 740
cortisol
–, blood 897
––, of men 897
–––, after rifampicin 897
–––, after streptomycin 897
–, plasma 740
––, diurnal variation 740
–, serum 183, 867
––, after insulin 867
––, after thyroliberin 867
––, quality control survey 183
coumarol
–, treatment 709
––, monitoring 709
counting
–, leukocytes 691
covariance selection 743
craniocerebral damage
–, creatine kinase 676
––, isoenzymes 676
creatine kinase
–, isoenzymes 676
––, craniocerebral damage 676
–, mitochondrial 679
––, radioimmunoassay 679
–, serum 675
––, *Duchenne* muscular dystrophy 675
creatine kinase-B
–, radioimmunoassay 678, 679
creatine kinase-BB
–, acute brain damage 668
–, macro form 677, 678
––, binding site 677, 678
–––, IgG 677, 678
creatine kinase-MB
–, acute myocardial infarction 676
–, determination 679
–, fractionation 678
creatinine
–, clearance 423
––, variation 423
–, physical exercise 651
–, serum 423
––, variation 423
–, true 385
––, serum 385
––, urine 385
–, urine 423, 705
––, variation 423
curve-fitting methods 105, 197
cuvette
–, specifications 445
––, listing 445
cystine aminopeptidase
–, placenta 631
cystinuria
–, screening 303
cytarabin
–, leukaemia treatment 690
––, ATP 690

dacarbazin
–, leukemia treatment 690
––, ATP 690
data
–, handling 947
–, structure 461
––, from different laboratories 461
definitive value 461
dehydroepiandrosterone
–, metabolism 747
––, placenta 747

dehydroepiandrosterone
–, plasma 117
––, determination 117
–, urine 741
––, determination 741
delipidation
–, serum 917
density
–, measurement 233
––, by mechanical oscillator technique 233
–, plasma 615
––, measurement 615
–––, and haemolysis 615
depression
–, 3-methoxy-4-hydroxy-phenylglycol 579
––, urine 579
depressive syndrome
–, function test 867
––, neuroendocrine 867
desacetylcefotaxime
–, serum 719
––, determination 719
–––, by HPLC 719
–, urine 719
––, determination 719
–––, by HPLC 719
detectability 78
diabetes
–, glycosaminoglycan, novel 672
–, lipoproteins 752
––, carbohydrate content 752
–, rat 901
diabetogenic action
–, thyroid hormone 738
diagnostic significance
–, tumour marker 821
dialysis
–, and lipoproteins 751
diethylstilboestrol
–, receptor binding 729
differentiation
–, leukocytes 647, 691, 692
––, *May-Grünwald-Giemsa* staining 691
digitonin
–, enzyme release 674
––, by rat heart 674
5α-dihydrotestosterone
–, and sex-hormone binding globulin 730
2,4-dinitrophenol
–, enzyme release 674
––, by rat heart 674
disc electrophoresis
–, proteins 53
––, urine 53
–––, value, diagnostic 53
diurnal variation see also variation
–, prostatic acid phosphatase 297
dopamine
–, plasma 367, 413
––, exercise 367
––, radioenzymatic assay 367
doxorubicin
–, leukaemia treatment 690
––, ATP 690
drugs
–, and alcohol 721
–, interference 385
Duchenne muscular dystrophy
–, creatine kinase 675
––, cord blood 675
––, serum 675
Dyggve-Melchior-Clausen syndrome
–, α_2-macroglobulin 67
––, serum 67

ECCLS
–, byelaws 637
––, revised 637
edema
–, hereditary angioneurotic 17
EDTA
–, lead elimination 163
elastase
–, leukocyte 699
––, determination 699
electroimmunoassay
–, apolipoprotein A-I 917
–, apolipoprotein A-II 917
elimination
–, lead 163
emergency analyses 693
emergency laboratory
–, information system 39
emission spectral analysis
–, aluminium 591
EMIT
–, methotrexate 169
encephalitis
–, viral 717
––, IgG 717
encephalomyelitis
–, experimental allergic 1
endometrium
–, steroid receptors 654
endotoxaemia
–, proteases 671
––, and protease inhibitors 671
enzyme
–, activities 649
––, physical exercise 649
–––, extracellular fluid 649
enzyme immunoassay
–, antiepileptics 720
–, heterogeneous 197
–, homogeneous 197
–, hormone determination 662
–, marker enzymes 197
–, mechanization 197
–, methotrexate 169
–, oestradiol-17β 664
–, oestrogens 327, 735
–, placental lactogen 735
–, pregnancy-specific β_1-glycoprotein 710
–, reliability 197
––, criteria of 197
–, testosterone 661
–, triiodothyronine 718
enzyme linked immunosorbent assay
–, peroxidase assay 345
enzymes
–, catabolism 679
––, two-compartment model 679
–, cellular 666
––, in extracellular space 666
–, determination 89
––, IFCC methods 89
–, differentiation 379
––, by lectins 379
–, elimination 666
enzymes
–, glycosaminoglycan depolymerizing 672
––, scoliotic children 672
–, markers 197
––, enzyme immunoassay 197
–, release 674
––, rat heart 674
–––, by Ca^{2+}-paradox 674
–––, by digitonin 674
–––, by 2,4-dinitrophenol 674
–, urine 686
––, kidney damage 686
enzyme tests
–, risk populations 763
––, identification 763
epidural analgesia
–, bupivacaine 351
––, determination 351
epinephrine
–, plasma 367, 413
––, exercise 367
––, radioenzymatic assay 367
epoxide hydrolase 775
epoxides
–, steroids 937
––, carcinogenesis 937
16α, 17α-epoxy-4-androsten-3-one 937
16α, 17α-epoxyoestratrienol 937
16α, 17α-epoxyprogesterone 937
errors
–, differentiation 433
erythrocytes see also blood, cord blood
–, cerebrospinal fluid 7
–, cord blood 771
––, catalase 771
––, glutathione peroxidase 771
––, superoxide dismutase 771
–, magnesium 257
–, maternal 771
––, catalase 771
––, glutathione peroxidase 771
––, superoxide dismutase 771
–, membrane 377
––, glycoproteins 377
–, oxygen dissociation curves 689
–, reference values 621
estrogen see oestrogen
ethanol
–, and γ-glutamyltransferase 237
–, microdetermination 722
ethosuximide
–, serum 339
–, gas chromatography 339
ethoxycumarine-O-deethylase 775
evaluation
–, instruments 693
––, principles 693
excretion
–, magnesium 257
exercise
–, catecholamine response 367
–, physical 649, 651, 652, 707, 708
––, enzyme activities 649
–––, extracellular fluid 649
––, HDL-cholesterol 707
exercise
–, physical
––, lactate 652
–––, formation kinetics 652
––, lipid metabolism 708
–––, in insulin deficiency 708
––, lipoproteins 652
––, metabolism 651
––, somatotropin release 708
–––, in pancreatectomized men 708
–, renin 367
extracellular fluid
–, enzyme activities 649
––, physical exercise 649
extracellular space
–, enzymes 666
––, cellular 666
extraction
–, bupivicaine 351
–, catecholamines 367
–, steroids 355

F-CB3
–, radioimmunoassay 711
factor VIII-related antigen
–, radioimmunoassay 711
faeces
–, blood 695
––, occult 695
–, chymotrypin 695
fast red TR 603
fenoterol
–, and hormones in pregnancy 748
–, tocolysis 687
ferritin
–, particle counting immunoassay 701
feto- see foeto-
fibrinolysis
–, determination 659
––, chromogenic substrates 659
–, regulation 657
fibronectin
–, body fluids 702, 797
––, laser nephelometry 702, 797
–, plasma 893
––, laser nephelometry 893
––, liver diseases 893
fibrinopeptide-A
–, radioimmunoassay 711
fibroblasts
–, alcohol dehydrogenase 699
–, aldehyde dehydrogenase 699
flow diagram
–, 5′-nucleotidase 333
–, uric acid 363
fluorometry
–, clobazepam 209
fluorouracil
–, leukaemia treatment 690
––, ATP 690
α-foetoprotein 821
–, particle counting immunoassay 701
folate
–, binding 715, 716
––, to cow's milk protein 715
––, to leukaemia serum protein 716
Friedewald formula 751
fucosyltransferase
–, serum 670
––, tumour therapy monitoring 670

function test
–, neuroendocrine 867
– –, for affective diseases 867

galactans
–, and lectins 376
galactosyltransferase II
–, tumour marker 763
gas chromatography
–, antiepileptics 720
–, antipyrine® 817
–, bupivacaine 351
–, carbamazepin 227
–, clobazepam 209
–, ethosuximide 339
–, 3-methoxy-4-hydroxy-phenylglycol 579
–, phenazone 817
–, phenobarbital 227
–, phenytoin 227
–, primidone 227
–, valproic acid 339, 497
gestosis
–, uric acid 749
glomerula
–, kidney 901
– –, isolated 901
– – –, metabolism 901
– – – –, and insulin 901
glucagon
–, and cAMP 909
– –, plasma 909
glucose
–, bivariate reference range 745
– –, CSF/serum 745
–, blood 129, 149, 255, 275
– –, determination 255, 275
– – –, kinetic 275
– –, and deproteinization 129
–, cerebrospinal fluid 275, 745
– –, cisternal 745
– –, determination 275
– – –, kinetic 275
– –, lumbar 745
– –, ventricular 745
–, determination 885
– –, blood 885
– – –, hemolysed 885
–, metabolism 901
– –, glomerula 901
–, monitoring 732
–, serum 49, 867
– –, after insulin 867
– –, after thyroliberin 867
– –, kinetic determination 49
– – –, hexokinase method 49
–, urine 275, 703
– –, determination 275
– – –, kinetic 275
glucose dehydrogenase
–, glucose determination 255
glucose-6-phosphate dehydrogenase
–, inhibition 49
– –, competitive 49
β-glucuronidase
–, amniotic fluid 611
– –, and sex of the child 611
glucuronidation 307
γ-glutamyltransferase
–, histochemistry 237
–, isoenzymes 673
–, liver 237
– –, and ethanol 237
–, plasma 237
– –, and ethanol 237
glutathione
–, depletion 713
– –, copper excretion 713
– –, zinc excretion 713
glutathione peroxidase
–, erythrocytes 771
– –, cord blood 771
– –, maternal 771
glutathione reductase
–, receptor model 724
goitre 909
granulocyte
–, migration 723
– –, and bee venom peptides 723
guaiac test 695
glycerol-2-phosphate
–, inhibitor 781
– –, 5′-nucleotidase 781
glycerophosphatides
–, and clotting tests 711
–, determination 711
– –, enzymatic 711
glycine
–, bile acid conjugation 407
– –, colorimetry 407
glycoconjugate-associated antigens
–, neoplastic cells 716
– –, and monoclonal cold agglutinins 716
glycoproteins
–, carbohydrate content 31
–, desialylated 31
– –, fate 31
– – –, in mouse circulation 31
–, erythrocyte membrane 377
–, interaction 379
– –, lectins 379
–, lymphocyte membrane 377
glycosaminoglycan
–, novel 672
– –, in diabetics 672
glycosaminoglycans
–, depolymerizing enzymes 672
– –, scoliotic children 672
–, rat 279
– –, liver 279
– –, serum 279

haematin D-575 689
haematocrit
–, fenoterol 687
–, reference values 621
–, verapamil 687
haematology
–, quality control 649
–, spin label method 648
haemodialysis
–, α_1-antitrypsin 241
– –, serum 241
haemoglobin
–, determination 689
– –, by alkaline haematin D-575 689
–, fenoterol 687
–, glycosylated 686
– –, chronic renal failure 686
–, reference values 621
–, verapamil 687
haemolysate
–, glucose 885
haemolysis
–, interference 615
– –, density measurement 615
–, reagent 255, 885
haemostasis 545
haemolyzing agent 255, 885
hair roots
–, alcohol dehydrogenase 699
–, aldehyde dehydrogenase 699
–, catechol-O-methyltransferase 672
HDL-cholesterol
–, determination 705
–, exercise, physical 707
–, method comparison 752
–, plasma 737
– –, and thyroid state 737
–, and pseudocholinesterase 684
HDL
–, apolipoprotein-A 917
heart
–, coronary disease 749
– –, lipoproteins 749
–, magnesium 257
–, rat 674
– –, enzyme release 674
– – –, by Ca^{2+}-paradox 674
– – –, by digitonin 674
– – –, by 2,4-dinitrophenol 674
hemo- see haemo-
heparin
–, action 710
–, plasma 658
– –, determination 658
– – –, clinical relevance 658
hepatic fibrosis 921
hereditary angioneurotic edema 17
heterogeneity
–, α_1-antitrypsin 13
high density lipoproteins 917
high performance liquid chromatography
–, aldosterone 742
–, cefotaxime 719
–, dehydroepiandrosterone 741
–, desacetylcefotaxime 719
–, 18-hydroxycorticosterone 742
–, testosterone 741
–, uric acid 702
high voltage electrophoresis
–, polyamines 698
histochemistry
–, γ-glutamyltransferase 237
hormone
–, determination 662
– –, enzyme immunoassay 662
hydrogen ion activity 829
18-hydroxycorticosterone
–, urine 742
– –, high performance liquid chromatography 742
– –, radioimmunoassay 742
hydroxylysyl glycosides
–, urine 287
hydroxyproline
–, urine 287
hyperlipoproteinaemia IV
–, bezafibrate therapy 750
– –, apolipoprotein C 750
hypermagnesaemia 257
hyperthyroidism 909
hypomagnesaemia 257
hypophysis
–, stimulation 867
hypothyroidism 909
–, congenital 755, 807
– –, screening 755, 807
hypoxanthine phosphoribosyl-transferase
–, variants 754

ICP-spectroscopy 591
IgA
–, cerebrospinal fluid 7, 245
– –, determination 245
IgE
–, serum 145
– –, method comparison 145
IgG
–, cerebrospinal fluid 7, 245
– –, determination 245
–, creatine kinase binding 677, 678
–, neurological diseases 717
–, particle counting immunoassay 701
–, protein-A binding 677
–, turbidimetric assay 501
IgM
–, cerebrospinal fluid 245
– –, determination 245
immune complex-like material
–, liver cirrhosis 717
–, septicaemia 717
immunoglobulins
–, serum 718
– –, determination 718
immunohistochemistry
–, parathyrin 739
immunoturbidimetry
–, protein determination 501
– –, measuring time interval 501
– –, reaction medium 501
imprecision 433
indometacin
–, and purine metabolism 754
indoxylphosphate
–, substrate 682
– –, phosphatase, acid 682
information system
–, emergency laboratory 39
inhibition
–, arylesterase 683
– –, by lanthanum 683
–, glucose-6-phosphate dehydrogenase 49
– –, by ATP 49
inhibitors
–, N-acetyl neuraminic acid 708
– –, of thromboxane synthesis 708
–, Cl esterase 17
–, monoamine oxidase 921
– –, β-aminopropionitrile 921
–, 5′-nucleotidase 781
–, sialyltransferase 271
instruments
–, evaluation principles 693
–, specifications 947
insulin
–, antibodies 313
–, deficiency 708
– –, lipid metabolism 708
– – –, and physical exercise 708
–, magnesium metabolism 257
–, metabolic activity 901
– –, glomerula 901
– – –, isolated 901
–, monitoring 732
–, neuroendocrine function test 867
–, plasma 313
–, urine 313

interferences
–, by drugs 385
–, gas chromatography 339
––, ethosuximide 339
––, valproate 339
–, haemoglobin 615
––, density measurement 615
–, proteins 697
––, by dextran 697
–, sialyltransferase assay 271
–, sucrose 23
––, protein pattern 23
–, turbidity 149
–, uric acid assay 59, 363
––, by ascorbate 363
interleukin-2 inducing protein 716
intestine
–, magnesium 257
ion exchange chromatography
–, catecholamine derivatives 567
––, of *o*-phthalaldehyde 567
–, orotic acid 293
–, polyamines 157
iron
–, plasma 714
––, method comparison 714
–, serum 147
––, method comparison 147
isoelectric focussing
–, enzymes 631
––, placental 631
–, polyacrylamide gel 13
––, protein separation 13
–––, effect of urea 13
–, transferrins 563
isoenzymes
–, amylase 669, 673, 680, 681
––, method comparison 681
–, creatine kinase 676, 677, 678, 679
–, γ-glutamyltransferase 673
–, lactate dehydrogenase 675
isotachophoresis
–, ascorbic acid 715

17-ketosteroids 741
kidney
–, chronic failure 686, 751
––, haemoglobin 686
–––, glycosylated 686
––, and lipoproteins 751
–, damage 686
––, enzymes 686
–––, urine 686
–, function 551
––, and pancreatic function test 551
–, glomerula 901
––, isolated 901
–––, metabolism 901
––––, and insulin 901
–, lesion 378
––, by neuraminidase 378
–, magnesium 257
–, maltase 685
–, *Tamm-Horsfall* glycoprotein 376
––, characterization 376
–, transplantation 241, 688
––, α_1-antitrypsin 241
–––, serum 241
––, pepsinogens 688
–––, serum 688
kinetic method
–, urea 706

kallikrein
–, urine 395
––, radioimmunoassay 395

lactate
–, cerebrospinal fluid 746
––, meningitis purulenta 746
–, physical exercise 652
lactate dehydrogenase
–, isoenzyme, sixth 675
––, in cardiogenic shock 675
–, reference values 305
––, age dependency 305
lanthanum
–, arylesterase inhibition 683
laser nephelometry 221, 245
–, fibronectin 702, 797, 893
–, lectin-glycoprotein interaction 379
LDL-cholesterol
–, *Friedewald* formula 751
–, method comparison 752
–, significance 751
lead
–, elimination 163
lectin
–, receptors 377, 384
––, leukemia cells 377
––, mammary gland 384
–, sponge 380
––, function 380
lectins
–, affinity chromatography 381
–, definition 373
–, enzyme differentiation 379
–, galactose specific 376
––, and galactans 376
–, interaction 379
––, glycoproteins 379
–, invertebrates 373
–, plants 373
–, protein binding 382
–, receptors 380
––, plexus chorioideus 380
–, specificity 373
–, structure 382
–, trypanosome characterization 383
–, vertebrates 373
Lesch-Nyhan syndrome
–, fibroblasts 754
––, metabolism 754
leukaemia
–, cells 377, 655, 690
––, ATP 690
–––, and cytotoxic substances 690
––, glucocorticoid receptors 655
––, lectin receptors 377
–, serum 716
––, folate binding 716
leukocytes
–, cerebrospinal fluid 7
–, counting 691
–, differentiation 647, 691, 692
–, elastase 699
–, reference values 621
lipids
–, metabolism 708
––, in insulin deficiency 708
–––, and physical exercise 708
lipoprotein lipase 753
lipoprotein X
–, catabolism 754

lipoproteins see also HDL, LDL, VLDL
–, carbohydrate content 752
–, and dialysis 751
–, method comparison 753
–, physical exercise 652
–, serum 749
––, coronary heart disease 749
β-lipotropin
–, preparation 736
–, determination 736
lithium
–, therapy 867
––, depressive patients 867
liver
–, carbon tetrachloride poisoning 111
–, cell injury 684
––, proteoglycan synthesis 684
–, cirrhosis 137, 717
––, corticosteroid binding globulin 137
––, immune complex-like material 717
––, sex hormone binding globulin 137
–, chronic diseases 737
––, sex hormones 737
––, thyroid hormones 737
–, diseases 893
––, fibronectin 893
–, fibrosis 921
––, and monoamine oxidase 921
–––, serum 921
–, foetal 748
––, adenylate cyclase 748
–––, and chorionic gonadotropin 748
–, γ-glutamyltransferase 237
––, and ethanol 237
–, glycosaminoglycans 279
–, injury 111, 279
––, proteins 111
–––, ribosomal 111
––, rat 279
–, magnesium 257
logit transformation 105
lung
–, diseases 13
––, α_1-antitrypsin 13
–, maturity 749
lymph nodes
–, magnesium 257
lymphocytes
–, membrane 377
––, glycoproteins 377
lysozyme 694

macro creatine kinase 677, 678
α_2-macroglobulin
–, chromogenic assay 671
–, plasma 221
––, determination 221
–, serum 67
––, *Dyggve-Melchior-Clausen* syndrome 67
–, serum/CSF gradient 671
magnesium
–, analysis 257
–, biochemical functions 257
–, distribution 257
––, extra-/intracellular 257
––, hormonal regulation 257
––, organelles 257
–, excretion 257
–, human body 257

magnesium
–, organs 257
–, physico-chemical state 257
––, extra-/intracellular 257
––, organelles 257
–, plasma 257
–, resorption 257
–, serum 257
–, transport 257
magnetic solid-phase radioimmuno-assay 706
maltase
–, determination 685
––, kidney preparation 685
––, urine 685
mammary carcinoma
–, oestrogen receptors 653
mammary gland
–, lectin receptors 384
malnutrition
–, protein 287
––, collagen metabolites 287
–––, urine 287
mass spectrometry
–, steroid epoxides 937
mast cell degranulating peptide
–, membrane interaction 723
matrix effects
–, precision 461
May-Grünwald-Giemsa staining 691
MCH
–, reference values 621
MCHC
–, reference values 621
MCV
–, reference values 621
mechanical oscillator technique
–, density 233
––, measurement of 233
mechanization
–, definition 455
–, enzyme immunoassay 197
medroxyprogesterone
–, receptor binding 729
melanoma
–, steroid binding 724
melatonin
–, synchronization of cell function 763
melittin 723
membrane
–, interaction 723
––, of mast cell degranulating peptide 723
meningitis purulenta
–, lactate 746
––, cerebrospinal fluid 746
mercury
–, determination 714
––, atomic absorption spectrometry 714
metabolism
–, physical exercise 651
metanephrine
–, thin-layer chromatography 413
methionine adenosyltransferase
–, schizophrenia 763
method comparison
–, amylase isoenzymes 681
–, blood alcohol 721
–, cholesterol 595, 752
–, IgE 145
–, iron 147, 714
–, lipoproteins 753
–, oestrogens 731
–, phosphatase, acid 681

method comparison
–, protein, total 697
–, statistical problems 433
–, thyrotropin 807
––, radioimmunoassay 807
–, uric acid 702
method dependence
–, assigned values 461
–, precision 461
method description 78
methotrexate
–, serum 169
––, enzyme immunoassay 169
3-methoxy-4-hydroxyphenylglycol
–, urine 579
––, gas chromatography 579
3-methoxytyramine
–, thin-layer chromatography 413
N1-methyl adenosine
–, urine 696
––, determination 696
methylation
–, enzymatic 413
––, catecholamines 413
N7-methyl guanosine
–, urine 696
––, determination 696
3N-methylhistidine
–, urine 756
––, and muscle catabolism 756
5-methyltetrahydrofolate
–, binding 715
––, to cow's milk protein 715
methyltrienolone
–, receptor binding 729
Michaelis constant
–, C1 esterase 17
mitochondria
–, creatine kinase 679
monoamine oxidase
–, determination 921
––, serum 921
–––, in hepatic fibrosis 921
–, inhibition 921
––, by β-aminopropionitrile 921
–, schizophrenia 763
morbus *Crohn*
–, fibronectin 797
mucopolysaccharidoses 67
multiple sclerosis
–, IgG 717
muscle
–, catabolism 755
––, 3N-methylhistidine 755
–––, urine 755
–, magnesium 257
myelin basic protein
–, enzyme immunoassay 345
myocardial infarction
–, creatine kinase-MB 676
–, enzymes 679

NADPH-cytochrome reductase 775
1-naphthol
–, coupling 603
––, with fast red TR 603
neonates
–, bilirubin 27
––, serum 27
–––, survey report 27
–, calcitonin 747
–, parathyrin 747
neoplastic cells
–, glycoconjugate-associated antigens 716
––, and monoclonal cold agglutinins 716
nephelometry
–, laser 221
––, α_2-antiplasmin 221
––, antithrombin III 221
––, α_2-macroglobulin 221
–, proteins 123, 245
––, cerebrospinal fluid 123, 245
nephrotic syndrome 381
neuraminidase
–, kidney lesion 378
neurophysins
–, preparation 736
neurosyphilis
–, IgG 717
neutron activation analysis
–, aluminium 403
–, phosphorus 403
nickel
–, inhibitor 781
––, 5'-nucleotidase 781
non-carboxylated coagulation factors 710
norepinephrine
–, plasma 367, 413
––, exercise 367
––, radioenzymatic assay 367
normal range
–, corticosteroid binding globulin 137
–, sex hormone binding globulin 137
normetanephrine
–, thin-layer chromatography 413
nucleic acids
–, metabolism 901
––, glomerula 901
5'-nucleotidase
–, inhibitors 781
–, spectrophotometric assay 781
–, serum 333
––, AutoAnalyzer II 333

oestradiol
–, and sex-hormone binding globulin 730
oestradiol-17β
–, enzyme immunoassay 664
oestradiol-fluorescein conjugate 727
oestriol
–, pregnancy 748
oestrogens
–, binding 724
––, melanoma 724
–, enzyme immunoassay 735
–, receptor 653, 728
––, capacity modulation 728
–––, by phosphatidyl choline 728
––, melanoma 653
–, serum 327
––, total 327
–––, enzyme immunoassay 327
–, urine 731
––, method comparison 731
oligosaccharides
–, affinity chromatography 381
–, separation 381
organization
–, laboratory 455
organ preservation
–, heavy water 747
organs
–, alcohol dehydrogenase 699
–, aldehyde dehydrogenase 699
ornithine transcarbamylase
–, deficiency 293
orotic acid
–, urine 293
––, determination 293
oscillator technique
–, density measurement 615
osmolality 829
osmotic concentration 829
outliers
–, recognition 433
oxygen
–, dissociation curves 689
––, determination 689
–, half saturation pressure 690
––, determination 690
–, partial pressure 829

palmitic acid
–, free 193
––, in bile 193
pancreas
–, diseases 763
––, trypsin 763
–, function test 551
––, oral 551
–––, N-benzoyl-*L*-tyrosyl-*p*-aminobenzoic acid 551
––––, and renal function 551
pancreatoctomy
–, and somatotropin release 708
––, physical exercise 708
paracetamol
–, copper excretion 713
–, zinc excretion 713
parathyrin
–, adsorption on surfaces 595
–, assay 664
–, localization 739
––, immunohistochemical 739
–, magnesium metabolism 257
–, radioimmunoassay 739, 789
––, homologous 789
––, influence of the tracer 739
–, serum 747
––, neonates 747
––, pregnancy 747
particle counting immunoassay
–, proteins 701
–, thyroxine 701
PCV
–, reference values 621
penicillamine
–, copper excretion 713
–, zinc excretion 713
pepsinogens
–, serum 688, 700
––, radioimmunoassay 688, 700
peptide hormones
–, reversed-phase chromatography 731
peptides
–, bee venom 723
––, and granulocyte migration 723
perfusion
–, placenta 747
–––, dehydroepiandrosterone metabolism 747
peroxidase
–, assay 345
––, enzyme immunosorbent assay 345
pH 829
phage test 1
phenazone
–, plasma 817
––, gas chromatography 817
phenobarbital
–, serum 227
––, determination 227
phenylalanine
–, metabolism 755
––, pregnancy 755
phenylketonuria 755
phenylpyruvic acid
–, blood 755
phenytoin
–, serum 227
––, determination 227
phosphatase, acid
–, determination 603
––, continuous 603
–, prostate 669, 681, 682
––, method comparison 681
–, prostate carcinoma 297
–, prostatic 627
––, determination 627
phosphatase, alkaline
–, stabilization 698
phosphatidyl choline
–, oestrogen receptor 728
––, capacity modulation 728
phospholipids
–, unsaturated 750
––, serum 750
phosphorus
–, bone biopsy 403
––, neutron activation analysis 403
phosphorylation
–, proteins 111
––, carbon tetrachloride poisoning 111
physical chemistry
–, quantities and units 829
pineal gland
–, synchronization of cell function 763
placenta
–, aminopeptidases 631
–, cystine aminopeptidase 631
–, dehydroepiandrosterone metabolism 747
–, proteins 821
placental lactogen 748
–, enzyme immunoassay 735
plasma see also blood, serum
–, adrenal steroid hormones 740
––, circadian variation 740
––, episodic variation 740
–, 5-androsten-3β, 17β-diol 117
–, α_2-antiplasmin 221
–, antipyrine® 817
––, gas chromatography 817
–, antithrombin III 221
–, bupivacaine 351
–, cAMP 909
––, after glycagon 909

plasma see also blood, serum
–, catecholamines 413, 431
––, conjugated 431
––, determination 413
–––, radioenzymatic 413
–, colloid osmotic pressure 233
–, corticotropin 740
––, circadian variation 740
––, episodic variation 740
–, cortisol 740
––, diurnal variation 740
–, C-peptide 313
–, C-reactive protein 701
–, dehydroepiandrosterone 117
–, density 615
––, and haemolysis 615
–, dopamine 367
–, enzymes 89
––, IFCC methods 89
–, epinephrine 367
–, fibronectin 797, 893
––, liver diseases 893
–, γ-glutamyltransferase 237
––, and ethanol 237
–, HDL-cholesterol 737
––, and thyroid state 737
–, heparin 658
––, clinical relevance 658
––, determination 658
–, insulin 313
–, iron 714
––, method comparison 714
–, α_2-macroglobulin 221
–, magnesium 257
–, norepinephrine 367
–, peptide hormones 731
––, separation 731
–, phenazone 817
––, gas chromatography 817
–, polyamines 157
–, proinsulin 313
–, protein 744
––, variation 744
–, proteins 615
––, determination 615
–––, oscillator technique 615
–, renin 367
–, β-thromboglobulin 656
––, clinical significance 656
––, determination 656
platelets
–, adenosine nucleotides 712
–, reference values 621
–, tetrahydro-β-carboline 133
–, tetrahydronorharmane 133
–, thromboxane synthesis 708
––, inhibition 708
–––, by N-acetyl neuraminic acid 708
plausibility
–, intervals 694
––, age 694
––, sex 694
plexus chorioideus
–, lectin receptors 380
podocytes
–, glomerular 381
––, and lectins 381
polyacrylamide gel electrophoresis
–, sample preparation 23
polyamines
–, plasma 157
––, determination 157
–, urine 157, 698
––, cancer patients 698
––, determination 157
polyarthritis, chronic
–, N-acetylglucosaminidase 53
––, urine 53
–, proteins 53
––, urine 53
–––, diselectrophoresis 53
potassium
–, urine 703
practicability 78
prealbumin
–, cerebrospinal fluid 7, 245, 746
––, determination 245
precision 69, 78, 433
–, matrix effects 461
–, method comparison 461
prednisone
–, leukemia treatment 690
––, ATP 690
pregnancy
–, calcitonin 747
–, complications therapy 748
––, and oestriol 748
––, and placental lactogen 748
––, and progesterone 748
–, parathyrin 747
–, phenylalanine 755
––, metabolism 755
–, progesterone 355
––, urine 355
pregnancy monitoring
–, pregnancy-specific β_1-glycoprotein 701
pregnancy specific β_1-globulin 821
pregnancy-specific β_1-glycoprotein
–, enzyme immunoassay 701
–, radioimmunoassay 701
–, pregnancy monitoring 701
–, tumour marker 701
pressure
–, colloid osmotic 233
–, partial 829
––, carbon dioxide 829
––, oxygen 829
primidone
–, serum 227
––, determination 227
processor
–, specifications 947
progesterone
–, pregnancy 748
–, radioimmunoassay 734
–, urine 355
––, determination 355
progestine
–, binding 724
––, melanoma 724
proinsulin
–, plasma 313
–, urine 313
prolactin
–, serum 867
––, after insulin 867
––, after thyroliberin 867
promegestone
–, receptor binding 729
prostate
–, carcinoma 297
––, phosphatase, acid 297
–, phosphatase, acid 627, 669, 681, 682
––, determination 627, 681, 682
–––, with indoxylphosphate 682
protease
–, inhibitors 671
––, in endotaxaemia 671
proteases
–, amniotic fluid 749
protein
–, cow's milk 715
––, folate binding 715
––, 5-methyltetrahydrofolate binding 715
–, determination 501
––, immunoturbidimetry 501
–, interleukin-2 inducing 716
––, serum 716
–, leukaemia serum 716
––, folate binding 716
–, malnutrition 287
–, metabolism 901
––, glomerula 901
–, plasma 744
––, variation 744
–, synthesis 111
––, liver 111
–––, after carbon tetrachloride 111
–, total 687, 696, 697
––, determination 696, 697
–––, method comparison 697
––, fenoterol 687
––, verapamil 687
protein-A
–, IgG binding 677
–, macro enzyme binding 677
protein binding
–, clobazepam 209
protein pattern
–, cerebrospinal fluid 23
––, and sample preparation 23
proteins
–, cerebrospinal fluid 123
––, nephelometry 123
–, lectin binding 382
–, placental 821
–, plasma 615
––, determination 615
–––, oscillator technique 615
–, stabilization 684
––, by heavy water 684
–, urine 53
––, disc electrophoresis 53
–––, value, diagnostic 53
proteoglycans
–, synthesis 684
––, in liver cell injury 684
pseudocholinesterase
–, and HDL-cholesterol 684
purines
–, metabolism 754
––, *Lesch-Nyhan* syndrome 754
putrescine
–, determination 157

quality
–, requirements 861
––, health care 861
quality control 855, 861
–, assessment of methods 78
–, biotransformation enzymes 775
–, haematology 649
–, internal 535
–, principles 69, 78
–, radioimmunoassay 215
–, survey, external 183, 511
––, cortisol 183
––, thyroxine 511
––, triiodothyronine 511
quality control
–, terminology 69
quantities 829

radioenzymatic assay
–, catechol-O-methyltransferase 672
––, hair root cells 672
radioimmunoassay
–, aldosterone 742
–, 5-androsten-3β, 17β-diol 117
–, anti-Factor VIII clotting activity 711
–, apolipoprotein A-I 917
–, apolipoprotein A-II 917
–, cAMP 732
––, inhibition by EDTA 732
–, chenodeoxycholic acid 491
–, cholic acid 491
–, confidence intervals 879
–, creatine kinase-B 678, 679
–, data 660
––, computer processing 660
–, dehydroepiandrosterone 117, 741
–, evaluation 215, 879
–, Factor VIII-related antigen 711
–, F-CB3 711
–, fibrinopeptide-A 711
–, homologous 789
––, parathyrin 789
–, 18-hydroxycorticosterone 742
–, kallikrein 395
–, magnetic solid-phase 706
–, pepsinogens 688
–, phosphatase, acid 681, 682
–, pregnancy-specific β_1-glycoprotein 701
–, progesterone 734
–, quality control 215
–, parathyrin 739
––, influence of the tracer 739
–, solid phase 585
––, parathyrin 585
–, somatostatin 734
–, standard curve 105
––, calculation 105
–, standardization 659
–, testosterone 741
–, thyrotropin 807
––, method comparison 807
–, thyroxine 571, 700
––, separation system 700
–, triiodothyronine 571
radioimmunosorbent test
–, IgE 145
radioreceptor assay
–, somatostatin 734
rapid tests
–, CSF diagnosis 7
––, sensitivity 7
rat
–, glycosaminoglycans 279
––, liver 279
––, serum 279
RBC
–, reference values 621
receptors
–, cAMP 656
––, tissue 656
–––, malignant 656
–, glucocorticoid 655
––, leukaemia cells 655
–, oestrogen 653, 728
––, capacity modulation 728
–––, by phosphatidyl choline 728
––, mammary carcinoma 653

receptors
–, steroid 654, 725, 726, 727, 728, 729
––, determination 725, 726, 727, 728, 729
––, endometrium 654
––, stability 726
–, studies 653
––, principles 653
reference material
–, coagulation 744
reference range
–, glucose 745
––, bivariate 745
–––, CSF/Serum 745
reference values
–, aluminium 591
––, serum 591
–, children 713
––, copper 713
–––, serum 713
–––, urine 713
––, zinc 713
–––, serum 713
–––, urine 713
–, haematology 621
–, lactate dehydrogenase 305
––, age dependency 305
reliability
–, criteria 197
––, enzyme immunoassay 197
renal damage
–, tubular 53
––, N-acetylglucosaminidase 53
–––, urine 53
––, disc electrophoresis 53
–––, urine proteins 53
renin
–, plasma 367
––, exercise 367
renin-angiotensin-aldosterone system
–, fenoterol 687
–, verapamil 687
resorption
–, magnesium 257
reversed-phase chromatography
–, peptide hormones 731
ribosomes
–, proteins 111
––, in liver injury 111
rifampicin
–, and cortisol 897
––, blood 897
–––, of men 897
–, and testosterone 897
––, blood 897
–––, of men 897
risk populations
–, identification 763
––, enzyme tests 763

sample
–, preparation 23
––, polyacrylamide gel electrophoresis 23
–, processor 692
sampling
–, specifications 947
Scherer medal 929
schizophrenia
–, methionine adenosyltransferase 763
„Schneller Block" 693
scintigraphy
–, immune 821
scoliosis
–, in children 672
––, glycosaminoglycans 672
screening
–, cystinuria 303
–, hypothyroidism 755, 807
––, congenital 755, 807
seminal plasma
–, fibronectin 797
septicaemia
–, immune complex-like material 717
serotonin
–, synchronization of cell function 763
serum see also blood, plasma
–, albumin 96
––, standard 96
–, aluminium 591
––, emission spectral analysis 591
–, antiepileptics 720
––, enzyme immunoassay 720
––, gas chromatography 720
–, bilirubin, total 27
––, neonates 27
–––, survey report 27
–, carbamazepine 227
–, carcinoembryonic antigen 175
–, calcitonin 747
––, neonates 747
––, pregnancy 747
–, cefotaxime 719
–, chenodeoxycholic acid 491
–, cholesterol 752
–, cholic acid 491
–, clobazepam 209
–, copper 712, 713
––, determination 712
––, reference values 713
–––, children 713
–, corticosteroid binding globulin 137
–, cortisol 183, 867
––, after insulin 867
––, quality control survey 183
––, after thyroliberin 876
–, creatine kinase 675, 676, 679
––, *Duchenne* muscular dystrophy 675
––, isoenzymes 676, 679
–––, acute myocardial infarction 676
–––, acute craniocerebral damage 676
–, creatinine 385, 423
––, determination 385
––, variation 423
–, deacetylcefotaxime 719
–, delipidation 917
––, high density lipoproteins 917
––, apolipoproteins 917
–, enzymes 89
––, IFCC methods 89
–, ethanol 722
––, microdetermination 722
–, ethosuximide 339
––, gas chromatography 339
–, fucosyltransferase 670
––, tumour therapy monitoring 670
serum see also blood, plasma
–, glucose 49, 867
––, after insulin 867
––, after thyroliberin 867
––, kinetic determination 49
–––, hexokinase method 49
–, glycosaminoglycans 279
–, IgE 145
–, immunoglobulines 718
––, determination 718
–, iron 147
–, lipoproteins 749, 752, 753
––, coronary heart disease 749
–, α_2-macroglobulin 67, 671
––, *Dyggve-Melchior-Clausen* syndrome 67
–, magnesium 257
–, methotrexate 169
––, enzyme immunoassay 169
–, monoamine oxidase 921
––, in hepatic fibrosis 921
–, 5'-nucleotidase 333
–, oestrogens 327
–, parathyrin 747
––, neonates 747
––, pregnancy 747
–, pepsinogens 688, 700
––, kidney transplantation 688
–, phenobarbital 227
–, phenytoin 227
–, phospholipids 705
––, unsaturated 750
–, primidone 227
–, prolactin 867
––, after insulin 867
––, after thyroliberin 867
–, protein 716
––, interleukin-2 inducing 716
–, sex hormone binding globulin 137
–, sex hormones 737
––, liver diseases 737
–, sialyltransferase 271
–, somatotropin 867
––, after insulin 867
––, after thyroliberin 867
–, standard 99
––, IFCC 74/1 99
–, thyroid hormones 737
––, liver diseases 737
–, thyrotropin 755, 867
––, hypothyroidism 755
–––, congenital 755
––, after insulin 867
––, after thyroliberin 867
–, thyroxine 571
––, radioimmunoassay 571
–, triiodothyronine 571
––, radioimmunoassay 571
–, urea 423
––, variation 423
–, uric acid 363, 702, 745
––, age dependency 745
––, sex dependency 745
–, valproate 339
––, gas chromatography 339
–, valproic acid 497
––, gas chromatography 497
–, zinc 712, 713
––, determination 712
––, reference values 713
–––, children 713
sex
–, of the child 611
––, and β-glucuronidase 611
–––, amniotic fluid 611
–, plausibility intervals 694
sex dependency
–, uric acid 745
––, serum 745
sex-hormone binding globulin
–, binding capacity 137
––, in liver cirrhosis 137
–, and 5α-dihydrotestosterone 730
–, and oestradiol 730
–, and testosterone 730
sex hormones
–, serum 737
––, liver diseases 737
shock
–, cardiogenic 675
––, lactate dehydrogenase isoenzymes 675
sialyltransferase
–, inhibitors 271
–, serum 271
––, determination 271
–––, interferences 271
significance
–, laboratory diagnosis 743
sodium
–, urine 703
somatostatin
–, catabolism 734
–, radioimmunoassay 734
–, radioreceptor assay 734
somatotropin
–, release 708
––, in pancreatectomized men 708
–––, and physical exercise 708
–, serum 867
––, after insulin 867
––, after thyroliberin 867
specifications
–, analysers 947
––, guidelines 947
specificity 78
spectrometers
–, specifications 445
––, listing 445
spectrophotometry
–, turbidity 149
––, influence of 149
spermidine
–, determination 157
spin label method
–, application in haematology 648
spleen
–, magnesium 257
spline functions 105
stability
–, alanine aminotransferase 521
standard
–, albumin 96
–, serum 99
––, IFCC 74/1 99
standard curve
–, radioimmunoassay 105
––, calculation 105
standardization
–, radioimmunoassay 659
standards 855
statistical methods 69
statistics
–, problems 433
––, in method comparison 433
stem cell population
–, monitoring 648
––, acute myeloid leukaemia 648

steroid
–, epoxides 937
––, carcinogenesis 937
–, extraction 355
–, microassays 661
–, receptor 725, 726, 727, 728, 729
––, determination 725, 726, 727, 728, 729
––, stability 726
stimulation
–, hypophysis 867
stomach
–, magnesium 257
streptomycin
–, and cortisol 897
––, blood 897
–––, of men 897
–, and testosterone 897
––, blood 897
–––, of men 897
streptozotocin 901
stress
–, lead elimination 163
substrates
–, chromogenic 557, 631
succinyl-ala-ala-val-4-nitroanilide
–, elastase substrate 699
sucrose
–, interference 23
––, protein pattern 23
sulfate
–, inorganic 651
––, physical exercise 651
survey report
–, bilirubin, total 27
––, serum 27
–––, neonates 27
superoxide dismutase
–, erythrocytes 771
––, cord blood 771
––, maternal 771
synchronization
–, cell function 763
synovial fluid
–, fibronectin 797

T-cell receptors
–, antigen specific 1
––, detection 1
––, isolation 1
T-lymphocytes 1
dTTPase
–, tumour marker 683
Tamm-Horsfall glycoprotein
–, kidney 376
temperature
–, enzyme assay 89
terminology
–, quality control 69
testosterone
–, blood 897
––, of men 897
–––, after rifampicin 897
–––, after streptomycin 897
–, enzyme immunoassay 661
–, and sex-hormone binding globulin 730
–, urine 741
––, determination 741
tetrahydro-β-carboline
–, platelets 129
tetrahydronorharmane
–, platelets 129
thin layer chromatography
–, androgens 117
–, clobazepam 209
thin layer chromatography
–, metanephrine 413
–, 3-methoxytyramine 413
–, normetanephrine 413
–, tetrahydronorharmane 129
thrombocytes
–, reference values 621
β-thromboglobulin
–, plasma 656
––, determination 656
––, clinical significance 656
thromboplastic activity
–, arterial walls 545
thrombosis 545
thromboxane
–, synthesis 708
––, inhibition by N-acetyl neuraminic acid 708
thyroid
–, function 909
––, and cAMP 909
–––, plasma 909
––––, after glucagon 909
–, state 737
––, and HDL-cholesterol 737
–, hormones 257, 737, 738
––, diabetogenic action 738
––, magnesium metabolism 257
––, serum 737
–––, liver diseases 737
thyroliberin
–, neuroendocrine function test 867
thyrotropin
–, radioimmunoassay 807
––, blood spot 807
–, serum 755
––, hypothyroidism 755
–––, cut-off point 755
thyroxine
–, particle counting immunoassay 701
–, quality control survey 511
––, external 511
–, radioimmunoassay 700
––, separation system 700
–, serum 571
––, radioimmunoassay 571
tissue
–, cAMP 656
––, receptors 656
tissue polypeptide antigen 821
tocolysis
–, fenoterol 687
–, verapamil 687
transferrin
–, determination 702
–, monoferric 563
––, isolation 563
transplant rejection
–, α_1-antitrypsin 241
––, serum 241
transport
–, magnesium 257
Trinder reagent
–, peroxidase assay 345
triiodothyronine
–, enzyme immunoassay 718
–, quality control survey 511
––, external 511
–, serum 571
––, radioimmunoassay 571
trypanosome
–, characterization 383
––, by lectins 383
trypsin
–, immunoreactive 763
––, pancreatic diseases 763
tuberculosis
–, treatment 897
––, and cortisol 897
––, and testosterone 897
tumour
–, fucosyltransferase 670
–, marker
––, carcinoembryonic antigen 175
––, diagnostic significance 821
––, dTTPase 683
––, galactosyltransferase II 763
––, pregnancy-specific β_1-glycoprotein 701
–, therapy monitoring 670
turbidimetry
–, proteins 245
––, cerebrospinal fluid 245
turbidity
–, interference 149
––, in spectrophotometry 149

UDP-glucuronyltransferase 307, 775
units 829
uranyl acetate
–, deproteinization 129
––, glucose determination 129
urea
–, clearance 423
–, determination 706
––, kinetic methods 706
–, physical exercise 651
–, serum 423
––, variation 423
–, urine 423, 703
––, variation 423
uric acid
–, determination 59
––, enzymatic 59
–––, interference 59
–––, SMAC 363
–, gestosis 749
–, physical exercise 651
–, serum 363, 702, 745
––, age dependency 745
––, high performance liquid chromatography 745
––, method comparison 702
––, sex dependency 745
urine
–, N-acetyl-β-D-glucosaminidase 53, 687
––, under amikacin therapy 687
––, under cisplatin therapy 687
––, determination 53
–––, value, diagnostic 53
–, adrenalin 567
–, alanine aminopeptidase 687
––, under amikacin therapy 687
––, under cisplatin therapy 687
–, aldosterone 742
–, analysis 703
––, Astra-8 Analyzer 703
–, ascorbic acid 715
––, determination 715
–––, isotachophoresis 715
–, ATP 686
––, bioluminescence assay 686
–, C-peptide 313
–, cefotaxime 719
–, chloride 703
–, clobazepam 209
urine
–, copper 713
––, reference values 713
–––, children 713
–, creatinine 385, 423, 703
––, determination 385
––, variation 423
–, dehydroepiandrosterone 741
–, desacetylcefotaxime 719
–, dopamine 567
–, enzymes 686
––, kidney damage 686
–, glucose 275, 703
–, 18-hydroxycorticosterone 742
–, hydroxylysyl glycosides 287
–, hydroxyproline 287
–, insulin 313
–, kallikrein 395
––, radioimmunoassay 395
–, maltase 685
–, 3-methoxy-4-hydroxy-phenylglycol 579
––, gas chromatography 579
–, N1-methyl adenosine 696
–, N7-methyl guanosine 696
–, 3N-methylhistidine 755
––, muscle catabolism 755
–, noradrenalin 567
–, oestrogens 731
––, method comparison 731
–, orotic acid 293
–, peptide hormones 731
––, separation 731
–, polyamines 157, 698
–, potassium 703
–, progesterone 355
––, pregnancy 355
–, proinsulin 313
–, proteins 53
––, disc electrophoresis 53
–––, value, diagnostic 53
–, protein, total 697
–, sodium 703
–, testosterone 741
–, urea 423, 703
––, variation 423
–, urokinase 557
–, zinc 713
––, reference values 713
–––, children 713
urokinase
–, urine 557

valproate
–, serum 339
––, gas chromatography 339
valproic acid
–, gas chromatography 497
value, diagnostic
–, N-acetylglucosaminidase 53
––, urine 53
–, clinical-chemical tests 743
–, disc electrophoresis 53
––, proteins 53
–––, urine 53
values
–, assigned 461, 855
––, definition 461
–, designated 855
–, reference method 461
variation
–, circadian 740
––, adrenal steroid hormones 740
–––, plasma 740
––, corticotropin 740
–––, plasma 740

variation
–, diurnal 740
––, cortisol 740
–––, plasma 740
––––, free 740
––––, total 740
–, episodic 740
––, adrenal steroid hormones 740
–––, plasma 740
––, corticotropin 740
–––, plasma 740
–, longitudinal 423
––, creatinine 423
–––, serum 423

variation
–, protein 744
––, plasma 744
verapamil
–, and hormones in pregnancy 748
–, tocolysis 687
vinblastin
–, leukaemia treatment 690
––, ATP 690
vincristin
–, leukaemia treatment 690
––, ATP 690

vitamin D
–, assay 765
VLDL 750

WBC
–, reference values 621
water
–, heavy 684, 747
––, organ preservation 747
––, protein stabilization 684

zinc
–, excretion 713

zinc
––, in rat bile 713
–––, and glutathione depletion 713
–––, and paracetamol 713
–––, and penicillamine 713
–, serum 712
––, determination 712
–––, atomic absorption spectrometry 712
–––, reference values 713
––––, children 713
–, urine 713
––, reference values 713
–––, children 713

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A New Principle of Thyroxine (T_4) and Triiodothyronine (T_3) Radioimmunoassay in Unextracted Serum Using Antisera with Binding Optima at Extreme pH Ranges[1])

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Summary: Thyroxine (T_4) or triiodothyronine (T_3) was covalently linked to bovine thyroglobulin (bTg) in a molar ratio of 25:1 (T_4:bTg) or 30:1 (T_3:bTg) using a water-soluble carbodiimide. The conjugates of T_4-bTg and T_3-bTg were used for immunisation of rabbits. After 6 booster-injections all rabbits immunized with T_4-bTg had antibody titers from 1:4000 or 1:8000, whereas the rabbits vaccinated with T_3-bTg had titers from 1:10000 to 1:40000. Two of the T_4-antisera revealed maximal T_4-binding between pH 3.8 and 4.2 with affinity constants of 7.8×10^9 l/mol and 6.0×10^9 l/mol. With these two antisera a simple, rapid, protein-independent T_4 radioimmunoassay at pH 4.0 in unextracted serum was established without using thyroxine binding globulin blocking agents. Precision of the T_4 radioimmunoassay is documented by an interassay coefficient of variation (CV) calculated from 10 different assays of 5.7% at a mean T_4 concentration of 43 μg/l T_4, 3.5% at 76 μg/l T_4 and 6.4% at 165 μg/l T_4. The intra-assay CV was calculated to be 3.7%, 2.8% and 4.7% at these concentrations. Accuracy of the T_4-RIA is documented by identical standard curves when T_4 standards were diluted in 1 g/l human serum albumin in phosphate buffer, in 40 g/l human serum albumin in phosphate buffer, or in T_4-free serum, and by recovery studies and dilution curves of hyperthyroid patients.

Three T_3 antisera with an optimal binding of T_3 at pH 4 and three T_3 antisera with a pH optimum in the pH range from 8.5 to 10.0 were obtained. A thyroxine binding globulin independent radioimmunoassay for T_3 could be established at pH 9.2 after thyroxine binding globulin in unextracted serum had been denaturated with 0.1 mol/l NaOH. The precision of this T_3 radioimmunoassay is documented by an inter-assay CV (n = 20) of 8.6% at a T_3 concentration of 0.91 μg/l T_3, 8.5% at a T_3 concentration of 1.720 μg/l T_3 and 5.8% at 4.150 μg/l T_3. The intra-assay CV calculated from 10 determinations was 5.8%, 3.1% and 3.4% at these T_3 concentrations. Accuracy of the T_3-RIA is documented by recovery studies, serum dilution curves and identical T_3 calibration curves, when T_3 standards were diluted either in 40 g/l human serum albumin in phosphate buffer or in T_3-free human serum.

Ein neues Prinzip für den Radioimmunassay von Thyroxin (T_4) und Triiodthyronin (T_3) im nicht extrahierten Serum: Verwendung von Antiseren mit Bindungsoptima bei extremen pH-Bereichen

Zusammenfassung: Mit wasserlöslichem Carbodiimid wurde sowohl T_3 als auch T_4 kovalent an Rinder-Thyroglobulin (bTg) in einem Verhältnis von 25:1 (T_4:bTg) bzw. 30:1 (T_3:bTg) gekoppelt. Mit diesen Konjugaten wurden je 6 Kaninchen immunisiert. Nach 6 Boosterungen im Abstand von 14 Tagen hatten die Kaninchen, die mit T_4-bTg immunisiert wurden, ausreichende Antikörper-Titer von 1:4000 bzw. 1:8000, die mit T_3-bTg immunisierten Kaninchen Antikörper-Titer von 1:10000 bzw. 1:40000. Zwei der T_4-Antiseren zeigten eine maximale T_4-Bindung im pH-Bereich von pH 3,8 bis pH 4,2 mit Assoziationskonstanten von $7,8 \times 10^9$ l/mol bzw. $6,0 \times 10^9$ l/mol. Mit diesen beiden T_4-Antikörpern konnte ein einfacher, schneller, Protein-unabhängiger T_4-RIA bei pH 4,0 im unextrahierten Serum ohne Einsatz von Thyroxin bindendes Globulin blockierenden Substanzen aufgebaut werden. Die Präzision dieses T_4-Radioimmunoassays konnte mit einem Interassay-Variationskoeffizienten, berechnet aus 10 verschiedenen Bestimmungen von 5,7% bei einer T_4-Konzentration von 43 μg/l T_4, 3,5% bei 76 μg/l T_4 und 6,4% bei 175 μg/l T_4 aufgezeigt werden. Der Intraassay-Variationskoeffizient, berechnet aus Zehnfach-Bestimmungen, betrug 3,7%, 2,8% und 4,7% bei diesen T_4-Konzentrationen. Identische T_4-Standardkurven wurden erhalten, wenn die T_4-Standards entweder in 1 g/l Humanserumalbumin in Phosphatpuffer, 40 g/l Humanserumalbumin in Phosphatpuffer oder T_4-freiem Serum gelöst waren. Damit wurde die Richtigkeit des T_4-RIA ebenso bewiesen, wie mit Wiederfindekurven und Serumverdünnungskurven, die jeweils auf der Standardkurve lagen.

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Wir erhielten drei T_3-Antiseren mit einem Bindungsoptimum bei pH 4,0 und drei T_3-Antiseren mit einem Bindungsoptimum im Bereich von pH 8,5 bis pH 10,0. Wir konnten einen T_3-RIA im unextrahierten Serum nach vorheriger Denaturierung von Thyroxin bindendem Globulin mit 0,1 mol/l NaOH und anschließender Inkubation bei pH 9,2 aufbauen. Die Präzision des T_3-RIA konnte mit einem Interassay-Variationskoeffizienten (n = 20) von 8,6% bei einer T_3-Konzentration von 0,91 μg/l T_3, 8,5% bei einer T_3-Konzentration von 1,72 μg/l T_3 und 5,8% bei 4,15 μg/l T_3 gezeigt werden. Der Intraassay-Variationskoeffizient, berechnet aus Zehnfach-Bestimmungen, betrug 5,8%, 3.1% und 3.4% bei den obengenannten T_3-Konzentrationen. Die Richtigkeit des T_3-RIA wurde mit Wiederfindekurven, Serumverdünnungskurven und identischen T_3-Standardkurven, wobei T_3-Standards entweder in 40 g/l Humanserumalbumin in Phosphatpuffer oder T_3-freiem Serum gelöst waren, bewiesen.

Introduction

The difficulties in radioimmunological determination of thyroxine (T_4) and triiodothyronine (T_3) in serum are mainly due to the binding of thyroid hormones to specific transport proteins in serum, mainly thyroxine binding globulin (TBG) (1), because the affinity constants of TBG to the thyroid hormones are often in the same order of magnitude as those of the antisera used (2). In recent years many modifications to radioimmunological determinations of thyroid hormones in serum have been published (3, 4, 5, 6). These have all been based upon two principles, using either the somewhat tedious procedure of extraction of T_4 and T_3 from human serum (7) or the more simple method, working with so-called TBG blocking agents mainly 8-anilino-l-naphthalene sulphonic acid (8, 9, 10, 11). These TBG blocking agents, however, also interact with the antisera used in the T_3 and T_4 assay (12), thus rendering this procedure inaccurate, because of varying TBG concentrations in serum. By modification of the immunisation technique we obtained antisera with binding optima for the thyroid hormones at acid or alkaline pH ranges. With these antisera a new type of T_4 and T_3 radioimmunoassay in unextracted serum could be established without the use of TBG blocking agents. Both radioimmunoassays are independent of endogenous TBG concentrations.

Materials and Methods

Reagents

Highly purified *L*-thyroxine (T_4), 3,5,3'*L*-triiodothyronine (T_3), 5,3',5'-triiodothyronine (rT_3) and tetraiodothyroacetic acid were a gift from the Henning GmbH Berlin. 3,5-diiodotyrosine, 3-monoiodotyrosine, 3,5-diiodothyronine and 3',5'-diiodothyronine were from the Fluka AG, Switzerland, bovine thyroglobulin and hydroxylamine from Sigma Chemicals Co., St. Louis, USA, donkey anti-rabbit-gamma-globulin precipitating serum (second antibody) from Wellcome Research Laboratories, Beckenham, England. Complete *Freund*'s adjuvant, diphtheria-pertussis-tetanus (DPT)-vaccine and human serum albumin from Behring AG, Frankfurt, Germany. Anion exchange cellulose (DE 32) were from Whatman Ltd., Springfield Mill Maidstone, England. All other reagents were p. a. substances from Merck AG, Darmstadt, Germany.

^{125}I-T_4 (specific activity about 3.7 TBq/g (100 Ci/g) was from Hoechst AG, Frankfurt, Germany, and was diluted either in 0.15 mol/l NaCl or in 0.1 mol/l HCl each containing 200 ml/l propylene glycol.

^{125}I-T_3 (specific activity of about 18.5 TBq/g ≙ 500 Ci/g) was also from Hoechst AG, Frankfurt, Germany. It was diluted in 0,05 mol/l phosphate buffer containing 1 g/l human serum albumin.

T_4-free serum was pooled from thyroidectomized patients treated longterm with thyrotropin suppressive doses of T_3. T_3-free serum was prepared by additional extraction with charcoal.

Preparation of T_4 and T_3 conjugates for immunisation

1 μmol of T_4 was dissolved in 100 μl of 0.1 mol/l NaOH and 50 μl dioxan, then 100 μl ^{125}I-T_4 (about 20,000 counts/min) purified by Sephadex G-25 chromatography (13), 15 mg bovine thyroglobulin dissolved in 300 μl 0.1 mol/l phosphate buffer pH 5.0 and 100 μl of 0.1 mol water soluble 1-ethyl-3-(3 dimethylaminopropyl) carbodiimide for covalent binding of T_4 to bovine thyroglobulin were added. After an incubation time of 24 hours at room temperature, the reaction was stopped by addition of 100 μl 0.1 mol/l hydroxylamine (14). The amount of T_4 linked covalently to bovine thyroglobulin (bTg) was calculated from the incorporation of the tracer amount of radioactivity into bTg after dialysis of the solution in a collodium bag (Sartorius Membranfilter GmbH, Göttingen, FRG). The molar ratio estimated was about 25:1 (T_4:bTg). T_3-bTg conjugate was obtained by an analogous procedure, the amount of T_3 incorporated to bovine thyroglobulin being approximately 30 mol T_3 per mol of bovine thyroglobulin.

Immunisation of rabbits

The conjugates of T_4-bTg and T_3-bTg were each dissolved in 3 ml 0.15 mol/l NaCl and emulsified with 2 ml of *Freund*'s complete adjuvant. These emulsions were each divided among 6 rabbits both for T_4-bTg and T_3-bTg. Sixty to eighty intradermal injections were made into the backs of the rabbits according to the method of *Vaitikaitis* (15). Each rabbit received a total of 0.8 ml of the emulsion, containing 2.5 mg bTg conjugate. In addition, diphtheria-pertussis-tetanus (DPT)-vaccine, 0.25 ml per rabbit, was injected at a separate site with the first two boosters. Injections were given at two week intervals. After the sixth booster the antibodies were harvested.

Precipitation procedures of antibody-bound thyroid hormones for radioimmunoassay

Precipitation of antibody-bound T_4 or T_3 for testing antibody titer at a physiological pH-range was performed with polyethylene glycol (M_r 6,000) (16). Polyethylene glycol (250 g) was dissolved in 1000 ml of 0.05 mol/l phosphate buffer pH 8.6. After incubation of 100 μl antiserum dilution with 100 μl of T_4-tracer (or T_3-tracer), 100 μl of 1 g/l bovine gammaglobulin in 0.15 mol/l NaCl and 300 μl of the 250 g/l polyethylene glycol solution were added. After vortexing, the tubes were centrifuged at 2000 *g* for 15 min, the supernatant aspirated off and the precipitate counted for radioactivity.

Because this precipitation method with polyethylene glycol only acts in the neutral pH range (16) and in addition is not specific for rabbit gamma-globulin, the precipitation of antibodies both at different pH ranges and also for radioimmunoassay was performed with a precipitating anti-rabbit-gamma-globulin donkey serum (second antibody) (17). This second antibody

reacts preferably at neutral pH range with specific rabbit antiserum, but it does not dissociate within 24 hours at either pH 3.0 or 11.0 when preincubated at pH 7.4 with the specific antiserum. Binding characteristics of the T_4 and T_3 antisera, which were tested in preliminary experiments, did not change by preincubation with the precipitating antibody. The solution of the complex at pH 7.4 is stable for at least 14 days at 4 °C in 0.15 mol/l NaCl.

Titration of the antisera

Antibody titer was calculated from a radioimmunological system at pH 7.4 in phosphate buffer 50 mmol/l. For T_4-antisera titration, 100 µl of ^{125}I-T_4 containing a total of 0.25 pg T_4 diluted in 0.25 mol/l NaCl containing 200 ml/l propylene glycol was added to 100 µl antiserum dilution in 1 g/l human serum albumin in phosphate buffer. After an overnight incubation, separation of antibody-bound and free hormone was performed with polyethylene glycol. For T_3-antisera, 100 µl ^{125}I-T_3 containing a total of 0.05 pg T_3 dissolved in 1 g/l human serum albumin in phosphate buffer, was added to 100 µl of T_3-antiserum dilution in 1 g/l human serum albumin in phosphate buffer. The antibody-bound and free hormone separation was performed with polyethylene glycol after an overnight incubation. Antibody titer was defined as the antiserum dilution added per tube which precipitated 50% of radioactivity added per tube.

pH-dependency of antibody binding with thyroid hormones

The antisera obtained were tested over a pH-range from pH 3 to 11 for their maximal tracer binding. The antiserum dilution was used which gave 50% tracer binding at pH 7.5. The following incubation scheme was used:

300 µl of a buffer solution were added to 100 µl of ^{125}I-T_4 and 100 µl of antiserum dilution preincubated with second antibody, each diluted in 0.15 mol/l NaCl. For the different pH values, 0.1 mol/l citrate buffer was used for pH 3.0 to 7.0, 0.1 mol/l tris-HCl-buffer for pH 7.5 to 9.0 and 0.1 mol/l glycine-NaOH buffer for pH 9.5 to 11.0. For unspecific binding (N) normal rabbit serum instead of specific antiserum was preincubated with second antibody. After an incubation time of 24 hours, when all of the antisera were in equilibrium, the tubes were centrifuged, the precipitate washed with 1 ml 0.15 mol/l NaCl and then counted for radioactivity.

Calculation of affinity constants

Affinity constants (K_a) of T_4 and T_3 antisera at the different pH-values were calculated from the calibration standard curves by *Scatchard* analysis (18). The T_4-standards in the range from 15 to 480 µg/l and the T_3-standards in the range from 0.25 to 8 µg/l were dissolved either in 1 g/l human serum albumin in phosphate buffer, 40 g/l human serum albumin in phosphate buffer or T_4- and T_3-free human serum.

Results

The pH-dependency of thyroid hormone binding of the T_4 and T_3 antisera

The six rabbits immunised with T_4-bTg conjugate developed antibody titers from 1:4000 to 1:8000 after 6 booster injections within 15 weeks. The 6 rabbits vaccinated with T_3-bTg conjugate showed titers from 1:10,000 to 1:40,000.

In respect to maximal T_4-tracer binding dependent on the pH of incubation buffer, the antisera could be divided into three groups (fig. 1). Two antisera (T_4-K_4 and T_4-K_6) demonstrated an optimal binding between pH 3.8 and 4.2, the antisera T_4-K_3 and T_4-K_8 had an optimum between pH 9 and 10, whereas the last group (T_4-K_5 and T_4-K_7) had no definite binding maximum in the pH range tested.

The T_4-antiserum T_4-K_5 could be separated on a DE-32 column (10 X 2 cm) into two gamma-globulin fractions using a linear gradient from 0.01 to 0.2 mol/l tris-phosphate buffer pH 8.2. One of the fractions bound most of the T_4-tracer at pH 4.0 whereas the other bound most of the T_4-tracer at pH 8.6 to pH 10.0 (fig. 1c).

In contrast to the T_4-antisera, none of the T_3-antisera had such a distinct pH dependency. Three of them showed a slight pH-optimum at pH 4, but up to pH 10 the binding of T_3 only fell slightly (to about 85%, taking binding at pH 4.0 as 100%). The remaining three antisera showed the opposite effect, binding maximally at pH 10.

Like the T_4-antiserum T_4-K_5, one of the T_3-antisera could be separated by DE-32 anion exchange chromatography into two antibody peaks, one with a prominent binding at pH 4, and the other at pH 9.0.

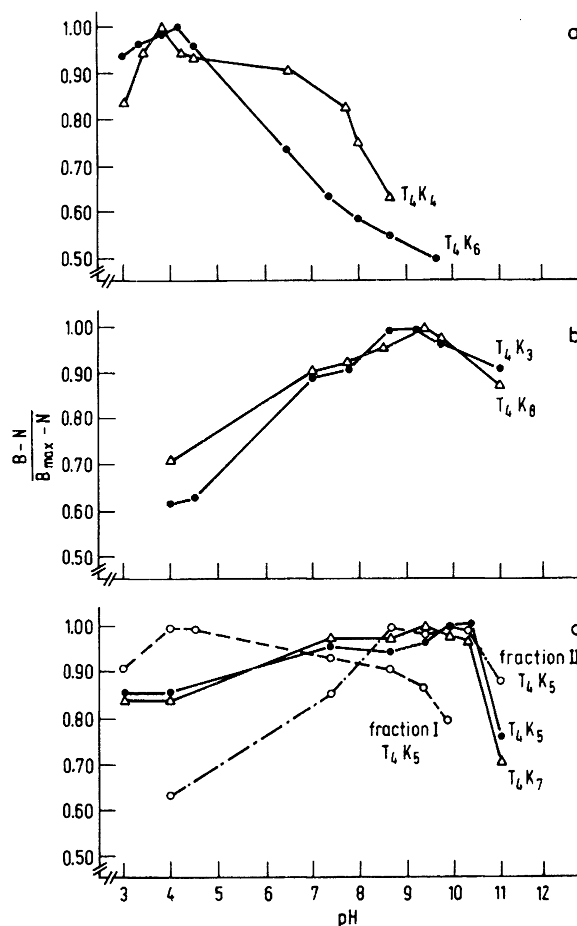

Fig. 1. Dependency of T_4 tracer binding of the T_4 antisera upon the values of the incubation medium. The maximal T_4 binding (B_{max} - N) of the antisera in group A was found to be in the range from pH 3.8 to pH 4.2, in group B from pH 9.0 to pH 10.0. Antisera of group C showed no clear cut pH dependency, however when the antiserum T_4-K_5 was fractionated on a DE-32 column into two antibody fractions, in fraction I the maximal binding of T_4 tracer was found at pH 4.0, whereas fraction II had an optimal binding in the range from pH 8.6 to pH 10.0.

Affinity constants of T_4 and T_3 antisera

The affinity constants (K_a) of T_4 antisera to T_4 and of T_3 antisera to T_3, respectively, were, as expected, highest in the pH range with maximal tracer binding (tab. 1).

The highest K_a of the T_3 antisera (T_3-K_9, T_3-K_{12}, T_3-K_{13}) with optimal binding between pH 8.0 and pH 9.5 were estimated to be 5×10^9 l/mol, 2×10^{10} l/mol and 6.5×10^9 l/mol respectively. The three antisera which bound maximally at pH 4.0 had affinity constants at this pH of 8.5×10^8 l/mol, 1×10^{10} l/mol and 1×10^9 l/mol.

Tab. 1. Dependency of the affinity constants (K_a) of four T_4 antisera upon the pH of the incubation buffer.

Antiserum	pH 4.0 K_a (l/mol)	pH 7.4 K_a (l/mol)	pH 10.0 K_a (l/mol)
T_4-K_4	7.8×10^9	4×10^7	2×10^5
T_4-K_6	6.0×10^9	2×10^7	1×10^5
T_4-K_3	1.0×10^6	2×10^7	2×10^9
T_4-K_8	2.0×10^5	8×10^6	3.5×10^9

Specifity of T_4 and T_3 antisera

The two T_4 antisera, T_4-K_4 and T_4-K_6, with binding optima at pH 4.0, and one T_3 antiserum, T_3-K_{12}, with an alkaline pH optimum, later used for a routine radioimmunoassay, were tested for crossreactivity with thyronine analogues, diluted in 1 g/l human serum albumin in phosphate buffer. As summarized in table 2 these antisera revealed high specifity.

Tab. 2. Crossreactivity of two T_4 antisera (T_4-K_4 and T_4-K_6) and T_3 antiserum (T_3-K_{12}) with thyronine derivates. The crossreactivity of the two T_4 antisera was found to be identical.

Compound	Crossreactivity in T_4-RIA (%)	in T_3-RIA (%)
L-Thyroxine	100	0.001
L-Triiodothyronine	0.1	100
Reverse triiodothyronine	3	0.003
Tetraiodothyroacetic acid	12	0.01
Diiodotyrosine	0.3	0.4
Monoidotyrosine	0.001	0.002
3',5'-Diiodothyronine	0.04	0.0004

T_4-radioimmunoassay in unextracted serum

Using these T_4-antisera with optimal binding at pH 4.0, a radioimmunoassay for routine purposes with 10 μl unextracted serum per incubation tube at this acid pH was developed without using TBG blocking reagents. The incubation schedule is summarized in table 3. Because of the high concentration of T_4, the antiserum dilution had to be only 1:800 per tube using 10 μl serum (calibration standard) to give a sensitive standard curve with a 50%-intercept at about 60 μg/l T_4.

Tab. 3. Incubation scheme of the T_4-RIA.

10 μl T_4 calibration standard (from 15 μg/l T_4 to 480 μg/l T_4) in 0.05 mol/l phosphate buffer pH 7.4 containing 40 g/l human serum albumin

or 10 μl serum

200 μl ^{125}I-T_4 in 0.1 mol/l sodium citrate buffer pH 3.8 containing 200 ml/l propylene glycol

100 μl T_4 antiserum (1:800) preincubated with second antibody (1:24), both diluted in 0.15 mol/l NaCl.

After an incubation time of 2 hours, when the reaction was at equilibrium, tubes were centrifuged for 10 min at 2000 *g*, the precipitate washed with 1 ml of 0.15 mol/l NaCl and after a further centrifugation the precipitate was counted for radioactivity. Calibration curves were calculated by spline function (19).

Quality control of T_4-radioimmunoassay

Precision of this assay is documented by the data summarized in table 4. Accuracy of T_4-radioimmunoassay was shown by independency of different protein contents of the samples: Identical standard curves were found when T_4-standards were diluted either in 1 g/l human serum albumin in phosphate buffer, 40 g/l human serum albumin in phosphate buffer or T_4-free human serum with a thyroxine binding globulin concentration of 18 mg/l (fig. 2). Furthermore, when sera of five hyperthyroid patients were diluted in 1 g/l human serum albumin in phosphate buffer, the T_4-values were on the standard curve (fig. 3). The recovery of a T_4-standard (80 μg/l) added to normal sera (n = 10) was found to be 98%. Total serum T_4-values determined in this radioimmunoassay (n = 32) of sera with different thyroxine binding globulin concentrations from 2.5 to 68 mg/l were found to be identical (r = 0.985; n = 32) when compared with T_4-concentrations determined by competitive protein binding analysis. In the latter method T_4 is extracted quantitatively from sera with simultaneous chromatography on alkaline Sephadex G-25 columns (7). The normal range of T_4-concentration in sera of 144 euthyroid patients was calculated to be 45 μg/l to 100 μg/l and is identical with the normal range of total T_4 which was determined by competitive protein binding analysis (7).

Tab. 4. Inter- and intraassay coefficient of variation (CV) of the T_4-RIA at three different T_4 concentrations in serum (n = 10).

T_4 concentration (μg/l)	Interassay CV (%)	Intraassay CV (%)
43	5.7	3.7
76	3.5	2.8
165	6.4	4.7

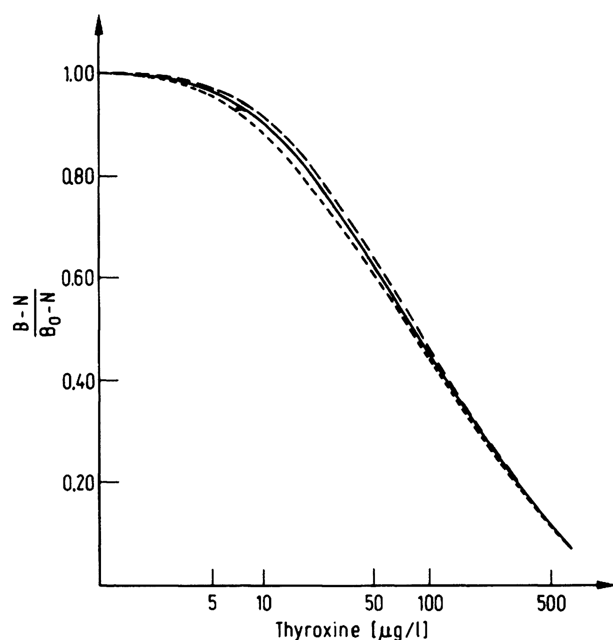

Fig. 2. T_4 calibration curves calculated from Spline function. T_4 standards were either diluted in 1 g/l human serum albumin in phosphate buffer (– – –), 40 g/l human serum albumin in phosphate buffer (------) or T_4-free human serum (——) with the thyroxine binding globulin concentration of 18 mg/l.

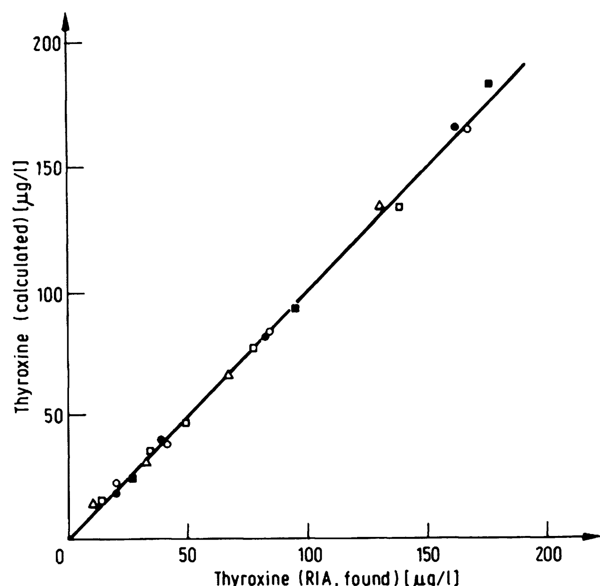

Fig. 3. Serum dilution curves. Sera of five hyperthyroid patients were diluted in a ratio of 1:2, 1:4, 1:8 with 1 g/l human serum albumin in phosphate buffer. T_4 values obtained from the T_4-RIA were identical to the calculated T_4 values (n = 20, r = 0.998, A = 0.0194, B = 995).
y = – 0.0194 + 0.995 x, r = 0.998.

T_3-RIA in unextracted serum

With the 3 antisera with a optimum of T_3 binding at pH 4.0 a T_3-radioimmunoassay was attempted along the lines of the T_4-radioimmunoassay. These T_3-antibodies were found to bind T_3 related to the different protein concentrations in the samples, as evidenced by serum dilution curves and the recovery of T_3-standards added to different sera. However, using the T_3-antisera with optimal binding at alkaline pH, a specific T_3-radioimmunoassay in unextracted serum could be established. The procedure is shown in table 5. Thyroxine binding globulin in serum is denaturated completely in 0.1 mol/l NaOH within 5 min at room temperature (20); the pH is then adjusted to 9.2 with 1.05 mol/l glycine-NaCl. Preincubated antiserum with the second antibody and the T_3-tracer was then added. After an incubation time of 4 hours the reaction was in equilibrium and the tubes were centrifuged for 10 min at approximately 2000 *g*. Standard curves and serum concentrations of T_3 were calculated by Spline function (19).

Tab. 5. Incubation scheme of the T_3-RIA.

25 µl T_3 calibration standard (from 0.25 µg/l T_3 to 8.00 µg/l T_3 in 40 g/l human serum albumin in phosphate buffer.
or 25 µl serum
200 µl 0.1 mol/l NaOH incubation for at least 5 min
100 µl 1.05 mol/l glycine-NaCl
100 µl ^{125}I-T_3 in 1 g/l human serum albumin in phosphate buffer
100 µl T_3 antiserum (1:32000) preincubated with second antibody (1:24) both diluted in 0.15 mol/l NaCl.

Quality control of the T_3-radioimmunoassay

The precision of the T_3-radioimmunoassay is documented by the data summarized in table 6. Accuracy of the assay is hown by identical standard curves, when T_3-standards were either deluted in 40 g/l human serum albumin in phosphate buffer or T_3-free serum with a thyroxine binding globulin concentration of 18 mg/l (fig. 4). Furthermore, when a T_3-standard (1.0 µg/l T_3 in 1 g/l human serum albumin in phosphate buffer) was added to human sera with different thyroxine binding globulin concentrations (from 9 mg/l to 23 mg/l) and different endogenous T_3-levels, the recovery was 100% (n = 37; r = 0.9985) (fig. 5). When sera of 5 hypertyhroid patients were diluted with 1 g/l human serum albumin in phosphate buffer, the T_3-concentrations were on the standard curve. The normal range of the T_3-concentration in sera of 144 euthyroid patients was calculated to be 0.8 µg/l to 1.6 µg/l.

Tab. 6. Inter- and intraassay coefficient of variation (CV) of the T_3-RIA at three different T_3 concentrations in serum (n = 20).

T_3 concentration (µg/l)	Interassay CV (%)	Intraassay CV (%)
0.910	8.6	5.8
1.720	8.5	3.1
4.150	5.8	3.4

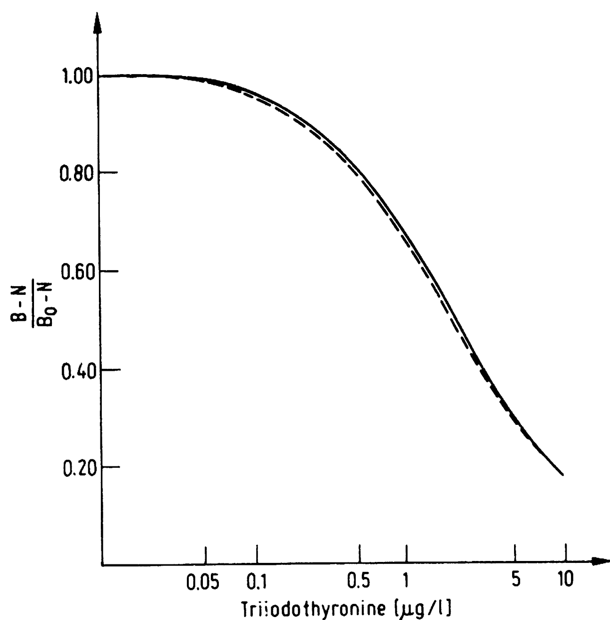

Fig. 4. T_3 calibration curves calculated from Spline function. T_3 standards were either diluted in 40 g/l human serum albumin in phosphate buffer (– – –), or T_3-free human serum with a thyroxine binding globulin concentration of 18 mg/l (——).

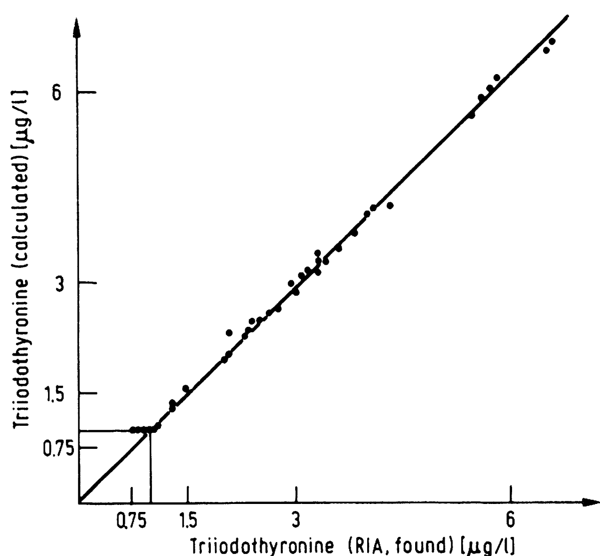

Fig. 5. Recovery study of a T_3 standard added to different human sera.
A T_3 standard (0.1 µg/l T_3 in 1 g/l human serum albumin in phosphate buffer) was added to 37 sera with different thyroxine binding globulin concentrations from 2.5 to 68 mg/l. The recovery was 100% (n = 37; r = 0.9985, A = 9.157, B = 0.958).
r = 0.9985, A = 9.157, B = 0.958

Discussion

Thyroglobulin is known to be a very suitable antigen for the production of specific antisera for linked haptens (14). Immunisation with isolated thyroglobulin which physiologically contains covalently bound T_4 and T_3 resulted in low titered T_4 and T_3 antisera (21). This study has demonstrated that monospecific antisera with sufficient titers can be obtained by immunisation with thyroglobulin enriched with covalently bound exogenous T_4 or T_3 respectively. Some of these antisera demonstrated the ability to bind the antigens with high affinity constants at either acidic of alkaline pH. This phenomenon may be explained by the mode of immunisation, using a high content of haptens coupled to a large glycoprotein molecule, in this case thyroglobulin, and short interval boosters resulting in multiclonal antibodies with different characteristics. The distribution of the antibody populations may vary widely in each of the inoculated animals. The individual selection of antibody clones with pronounced pH dependency was more distinct in the rabbits immunized with T_4/bTg conjugate compared with those immunized with the T_3-bTg conjugate.

Until now, T_4 and T_3 radioimmunoassay in unextracted serum could be only performed by using thyroxine binding globulin blocking agents such as 8-anilino-1-naphthalene sulphonic acid and merthiolate (6). These agents showed a displacement of T_4 and T_3 not only from thyroxine binding globulin but also from the antibodies (12), therefore the accuracy of these assays may be influenced by the differences in endogenous thyroxine binding globulin concentrations in the serum samples.

As demonstrated in the last external quality control survey (20) compared to the survey in 1976 (21), the quality of T_4-RIA kits has been improved. However, using two commercial T_4-RIA kits (Gamma Coat, Travenol and ^{125}I-T_4-RIA, Poemix, Biosigma) and sera containing diminished thyroxine binding globulin (TBG) levels, we found high T_4 concentrations with elevated T_4/TBG ratios, which disagreed with clinical findings and thyroliberin stimulation tests. Therefore dilutions of sera with elevated, normal and diminished thyroxine binding globulin were made from the T_4-free serum provided with the kits. Whereas T_4 dilution curves of sera with normal and elevated thyroxine binding globulin levels yielded satisfactory results, the sera of patients with diminished thyroxine binding globulin levels gave inaccurate T_4 dilution curves (tab. 7). This

Tab. 7. Determination of total T_4 in sera with different thyroxine binding globulin (TBG) levels undiluted and in dilutions with T_4-free serum, using two commercial T_4-RIA kits (kit A: Gamma Coat, Travenol, kit B: ^{125}I-T_4-RIA-Poemix, Biosigma).

		Elevated		Normal		Dininished	
	TBG levels (mg/l)	39	34	20	19	4	6
	T_4 undiluted	185	168	102	116	61	30
Kit A	(µg/l) 1:2	111	93	56	62	18	35
	1:4	63	49	36	38	18	22
	T_4 undiluted	201	172	95	115	52	21
Kit B	(µg/l) 1:2	102	93	49	54	14	30
	1:4	60	48	31	32	12	19

demonstrates dependence of T_4 analytical results on the endogenous thyroxine binding globulin levels in these methods. As demonstrated by *Gershengorn* et al. (20), thyroxine binding globulin irreversibly changes its tertiary structure at a pH lower than pH 4.5 and above pH 11.5, and the ability to bind thyroid hormone is lost. With the availability of T_4 antibodies with a binding optimum at pH 4.0, a simple, specific and protein-independent radioimmunoassay could be established, because the endogenous T_4 binding proteins showed no influence on T_4 antibody binding in this pH range. This is documented by identical standard curves of T_4 diluted either in 1 g/l human serum albumin in phosphate buffer, 40 g/l human serum albumin in phosphate buffer or T_4-free human serum with the endogenous thyroxine binding globulin concentrations of 18 mg/l thyroxine binding globulin, by recovery studies and by dilution curves of sera with different thyroxine binding globulin concentrations.

In rabbits, the selection of T_3 antibodies with a binding optimum in an acid pH range was less pronounced compared with the T_4 antisera. A T_3 radioimmunoassay in unextracted sera at pH 4.0 according to the T_4-RIA without prior separation of the T_3-antibody populations by anion exchange chromatography, did not fulfill criteria of accuracy. However, using the T_3 antisera with alkaline binding optimum, a thyroxine binding globulin independent T_3 radioimmunoassay could be established without thyroxine binding globulin blocking agents, after a prior denaturation of thyroxine binding globulin with 0.1 mol/l NaOH.

Preincubation of the specific antibody with precipitating antibody (second antibody) did not influence the characteristics of the specific antibody. This procedure is necessary when the radioimmunoassay is performed at extreme pH ranges where the second antibody does not bind the specific antibody; it also represents a simplification of the assay procedure.

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