METABOLITES OF CATECHOLAMINES IN THE CEREBROSPINAL FLUID

D. F. SHARMAN

Agricultural Research Council, Institute of Animal Physiology,
Babraham, Cambridge, CB2 4AT

THE MAJOR ACIDIC METABOLITES OF THE CATECHOLAMINES IN CEREBROSPINAL FLUID

FIFTEEN years ago, G.W. Ashcroft and I were working together under the tutelage of T. B. B. Crawford in the Department of Pharmacology in the University of Edinburgh. We had found that a substance, behaving like 5-hydroxyindol-3-ylacetic acid (5-HIAA) on paper chromatography and showing chemical reactions similar to this metabolite of 5-hydroxytryptamine (5-HT), was present in brain tissue (SHARMAN, 1960). We then examined human cerebrospinal fluid for the presence of 5-hydroxyindoles and found that we could detect a substance or substances having similar fluorescence characteristics to those exhibited by 5-hydroxyindole compounds. In order to try to identify this material we obtained relatively large volumes of cerebrospinal fluid from patients suffering from hydrocephalus and who were undergoing ventricular drainage. These samples of cerebrospinal fluid were found to contain a concentration of 5-hydroxyindoles which was higher than normal (ASHCROFT and SHARMAN, 1960). The work of Armstrong and his colleagues on the phenolic acids of human urine (ARMSTRONG, SHAW and WALL, 1956) led us to apply similar analytical techniques to cerebrospinal fluid. In the first samples we usually found four substances which gave a clear colour reaction with diazotised *p*-nitroaniline. One of these was found to be salicylic acid. Paper chromatography of an extract of cerebrospinal fluid from a patient who was *not* receiving acetylsalicylic acid indicated the presence of three phenolic substances. The R_f values, colour and fluorescence reactions which were applied to these substances showed that Spot 1 was 5-hydroxyindol-3-ylacetic acid. It was concluded that Spot 2 was probably 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid; HVA) a metabolite of 3,4-dihydroxyphenylethylamine (dopamine) but the further examination of this substance was not carried out until later. Spot 3 appeared to be isographic on paper chromatograms with 4-hydroxy-3-methoxymandelic acid (vanillyl mandelic acid; VMA) a metabolite of noradrenaline. However, there was one reaction which distinguished Spot 3 from this metabolite. When VMA is reacted with 2,6-dichloroquinonechloroimide (Indophenol reaction; GIBBS, 1927) an intense blue colour is developed, but Spot 3 gave no reaction with this reagent. It is now thought that this substance is *p*-hydroxyphenyllactic acid SJÖQUIST and ÄNGGÅRD, 1972). This could be a product of transamination of tyrosine.

In 1963, fluorimetric methods for the estimation of HVA were published (ANDÉN, ROOS and WERDINIUS, 1963a; SHARMAN, 1963) and the former authors clearly demonstrated the presence of HVA in cerebrospinal fluid. These reactions are based

on the oxidation of HVA to form a fluorescent dimer, 2,2¹-dihydroxy-3,3¹-dimethoxy-biphenyl-5,5¹-diacetic acid (CORRODI and WERDINIUS, 1965). The procedure of ANDÉN *et al.*, (1963a) has formed the basis for most of the methods now in use for the fluorimetric analysis of HVA in the cerebrospinal fluid. HVA is extracted from a salt saturated, deproteinised extract of cerebrospinal fluid at an acid pH, into an organic solvent such as di-ethylether, ethylacetate or *n*-butylacetate. The HVA is then back extracted into an alkaline buffer solution. ANDÉN *et al.* (1963a) used 0·05M-tris (hydroxymethyl) amino methane, pH 8·5, GERBODE and BOWERS (1968) used 0·5M-sodium phosphate buffer pH 8·5 and borate buffer may also be used (PULLAR, personal communication). The choice of buffer is important since the conditions for the development of the fluorescence are critical. Small amounts of other ions in the buffer solution can greatly reduce the intensity of the fluorescence produced or completely prevent its development. KORF, VAN PRAAG and SEBENS (1971) have tried to improve the extraction procedure by using a column of Sephadex G10 to extract HVA from cerebrospinal fluid, a procedure which allows the estimation of 4-hydroxy-3-methoxyphenylethyleneglycol (MHPG), a metabolite of noradrenaline, to be made on the same sample. The HVA is eluted from the Sephadex G10 with 0·5M-sodium phosphate buffer. The fluorescent dimer is formed by oxidation in a very dilute (1–50 μg/ml) solution of potassium ferricyanide. The oxidation reaction is terminated and the fluorescent product stabilised with a reducing agent, usually cysteine. The fluorescence shows a maximum activation wavelength of about 320 nm and a maximum fluorescence wavelength of about 430 nm. (The reported values for these wavelengths are uncorrected instrumental values.) The fluorescence developed from the apparent HVA in cerebrospinal fluid shows similar maxima.

The specificity of the reaction is reasonable. GJESSING, VELLAN, WERDINIUS and CORRODI (1967) have shown that several substances having the vanyl (i.e. 4-hydroxy-3-methoxyphenyl) group in their structure will give rise to a similar fluorescence. Among these vanyllactic acid must be considered since it is found in the urine of patients treated with L-3,4-dihydroxyphenylalanine (L-dopa) and would be extracted, if present in the cerebrospinal fluid of such patients. When other phenolic acids including *p*-hydroxyphenylacetic acid are oxidised with hydrogen peroxide in the presence of peroxidase, a fluorescent derivative is produced (GUILBAULT, BRIGNAC and JUNEAU, 1968) but this acid and related compounds gave little or no fluorescence when oxidised with ferric chloride (SHARMAN, 1963) and SATO (1965) has shown that *p*-hydroxyphenylacetic acid yields less than 2 per cent of the fluorescence seen when HVA is treated by the method of ANDÉN *et al.*, (1963a).

Recently an isomer of HVA, 3-hydroxy-4-methoxyphenylacetic acid (homoisovanillic acid; iso-HVA) has been demonstrated to be present in cerebrospinal fluid, a finding which may have far reaching implications. It is known that, *in vitro*, the enzyme catechol-*O*-methyl transferase, which is responsible for the introduction of the methyl group into position 3 of the phenyl group of catecholamines and their catechol metabolites can also methylate the phenolic hydroxyl group at the 4 position. About 10 per cent of the substrate is methylated at position 4 by the enzyme extracted from rat liver (AXELROD, 1966). MATHIEU, REVOL and TROUILLAS (1972) and MATHIEU, CHARVET, CHAZOT and TROUILLAS (1972) have separated HVA and iso-HVA by paper chromatography of the products formed by coupling these two acids with diazotised *p*-nitroaniline, since it is difficult to separate the parent compounds

by the usual analytical procedures. However, iso-HVA does not give rise to a fluorescence if subjected to the method of ANDÉN *et al.* (1963) for the fluorimetric assay of HVA (KIRSCHBERG, COTE, LOWE and GINSBURG (1972) and MATHIEU, CHARVET, CHAZOT and TROUILLAS (1972) have shown that the fluorimetric assay of HVA in cerebrospinal fluid gives results which are comparable with the values obtained by their colorimetric procedure after separation of the two isomers. Iso-HVA can depress the fluorescence yield from HVA as can other acids which might be present in cerebrospinal fluid (KIRSCHBERG *et al.*, 1972). In practice, the other acids normally present in extracts appear not to affect the development of the fluorescence to any great extent, but this should be checked particularly if samples are taken from subjects which have been treated with large amounts of catecholamine precursors or metabolites. There has been little use made of gas–liquid chromatography for the routine estimation of HVA in cerebrospinal fluid. SJÖQUIST and ÄNGGÅRD (1972) have described a method for the estimation of HVA in human cerebrospinal fluid using heptafluorobutyryl derivative of the methyl ester of HVA. This substance was detected after gas chromatography by electron capture or by the multiple ion detector of a mass spectrometer. When the latter method of detection was used, the deuterated-methyl ester of HVA could be used as a standard. The observations of these authors confirm the presence of HVA in human cerebrospinal fluid. The derivative described by DZIEDZIC, BERTANI, CLARKE and GITLOW (1972) has possibilities in this respect. These authors have shown that the hexafluoro-isopropyl ester of 4-trifluoroacetyl-HVA has excellent electron capture properties. At Babraham, we have used this derivative in a method for the estimation of HVA in nervous tissues and have shown that it can be applied to cisternal cerebrospinal fluid from the pig.

In our early experiments we did not detect any 3,4-dihydroxyphenylacetic acid (DOPAC), another known metabolite of dopamine, on the paper chromatograms, a result similar to that obtained by ANDÉN, ROOS and WERDINIUS (1963b). However, ASHCROFT, CRAWFORD, DOW and GULDBERG (1968) showed that DOPAC was present in extracts of dog cerebrospinal fluid and used the fluorescence which is developed when DOPAC is condensed with 1,2-diaminoethane to estimate its concentration. In fluid taken from the lateral ventricle of the dog, the concentration of DOPAC was 237 ng/ml and that of HVA 2298 ng/ml. In normal human lumbar cerebrospinal fluid the concentration of DOPAC is near the limit of the sensitivity of the fluorimetric method of assay but WATERBURY and PIERCE (1972) have demonstrated the presence of this metabolite in human lumbar cerebrospinal fluid by a gas chromatographic method involving the formation of the trimethylsilyl derivative of its methyl ester combined with mass spectrometry. The gas chromatographic method for HVA in which the trifluoroacetyl-hexafluoro-iso-propyl derivative is made can also be used for DOPAC. We have found a small peak at the appropriate relative retention time for the derivative of DOPAC on our records of extracts from pig cerebrospinal fluid but we have not yet confirmed that this peak represents DOPAC only.

The presence of either of two possible acidic metabolites of noradrenaline 3,4-dihydroxymandelic acid (DOMA) and VMA in cerebrospinal fluid has not been unequivocally demonstrated.

ALCOHOL METABOLITES OF CATECHOLAMINES IN CEREBROSPINAL FLUID

During the past ten years evidence has accrued to show that the major metabolite of noradrenaline in the brain is 1-(4-hydroxy-3-methoxyphenyl) ethane-1,2-diol

(4-hydroxy-3methoxyphenylethyleneglycol, MHPG, MOPEG). The formation of this metabolite was demonstrated in cat brain *in vivo* by MANNARINO, KIRSHNER and NASHOLD (1963) and in rabbit brain *in vitro* by RUTLEDGE and JONASON (1967). In 1968, SCHANBERG, SCHILDKRAUT, BREESE and KOPIN found that in the rat brain, the major metabolite formed from radioactively labelled 1-(4-hydroxy-3-methoxyphenyl)-2-aminoethan-1-ol (normetanephrine, NM) was the ethereal sulphate conjugate of MHPG. The presence of MHPG-sulphate in the brains of some other mammalian species in addition to the free compound was demonstrated by SCHANBERG, BREESE, SCHILDKRAUT, GORDON and KOPIN (1968) who also showed that MHPG and its sulphate conjugate was present in human cerebrospinal fluid.

A method for the estimation of MHPG in urine, using gas chromatography and electron capture of the trifluoroacetyl derivative of MHPG was described by WILK, GITLOW, CLARKE and PALEY (1968) and this method was applied by SCHANBERG, BREESE, SCHILDKRAUT, GORDON and KOPIN (1968) to human cerebrospinal fluid. In this method, the conjugated MHPG is hydrolysed by incubation with a sulphatase preparation and the MHPG is absorbed onto a column of Biorad AG 1 $\times$ 4 exchange resin in the chloride form. The MHPG is eluted with water and then extracted into ethylacetate. After evaporating to dryness the extract is treated with trifluoroacetic anhydride to yield the tri-trifluoroacetyl derivative of MHPG. More recently the absorption and elution step has been omitted by some workers for the analysis of cerebrospinal fluid (WILK, DAVIS and THACKER, 1970; GORDON and OLIVER, 1971). Another derivative of MHPG, acetyl-di-trifluoroacetyl-MHPG, has been used, by BOND (1972) to estimate MHPG in urine and cerebrospinal fluid. This author ran into difficulties when using the method of WILK *et al.* (1967) and found that acetylation of MHPG before reacting with trifluoracetic anhydride gave satisfactory results. Acetylation of MHPG and of 1-β,4-dihydroxyphenyl)ethane-1,2-diol (DHPG, DOPEG) in aqueous solution followed by extraction and the formation of di-heptafluorobutyryl derivatives has been used to measure the concentrations of these two metabolites in brain tissue (SHARMAN, 1969; CEASAR and SHARMAN, 1972). The acetylation procedure imparts a greater stability to the compounds and enables more rigorous separation procedures to be used. The responses of the gas chromatograph, perhaps because of the exquisite sensitivity of such methods, are just as prone to be misinterpreted as those of the fluorimeter, particularly when preceded only by a simple solvent extraction. However, when the amounts of the substances to be estimated are very small, it may only be possible to use such a simple extraction procedure in order to avoid excessive losses. The use of an ion exchange resin in the methods for the estimation of MHPG (WILK *et al.*, 1967; SCHANBERG, BREESE, SCHILDKRAUT, GORDON and KOPIN, 1968; BOND, 1972) improves the specificity of the method and PULLAR (personal communication) has found that a small column of the anion exchange resin in the hydroxide form can be used to great advantage for the extraction of MHPG in his method which also uses gas chromatography and electron capture detection of the acetyl-di-trifluoroacetyl derivative. WATERBURY and PIERCE (1972) have been able to identify MHPG in human cerebrospinal fluid by gas chromatography of its trimethylsilyl derivative.

Fluorimetric methods for the estimation of MHPG have also been used. KORF, VAN PRAAG and SEBENS (1971) have isolated MHPG by means of absorption on to and elution from a column of Sephadex G10 in their method which also permits the

estimation of 5-HIAA and HVA in the same sample of cerebrospinal fluid. Potassium ferricyanide solution and a mixture of 1,2 diaminoethane, ammonium chloride and ammonium hydroxide is added to the eluate and the mixture is heated for 15 min after which a solution of cysteine is added. This procedure develops a fluorophore from MHPG, the fluorescence of which is measured, with activating light set at 400 nm wavelength, at a wavelength of 500 nm. A fluorimetric method for the estimation of the sulphate conjugate of MHPG has been described by MEEK and NEFF (1972) but there have been no reports of its application to cerebrospinal fluid. The above methods clearly demonstrate that MHPG is a normal constituent of cerebrospinal fluid and if used correctly give accurate estimates of its concentration. The possibility that 1-(3-hydroxy-4-methoxyphenyl) ethan-1,2-diol(ISO-MHPG) might be present in cerebrospinal fluid has been considered by MATHIEU *et al.* (1972) but as yet this substance has not been shown to be present.

The presence in or absence from cerebrospinal fluid of alcohol metabolites of dopamine, i.e. 2-(3,4-dihydroxyphenyl)ethan-1-ol (DHPE, DOPOL) and 2-(4-hydroxy-3-methoxyphenyl)ethan-1-ol (MHPE, MOPOL) is at present a subject for discussion. WATERBURY and PIERCE (1972) have reported the presence of DHPE and MHPE in human cerebrospinal fluid on the basis of the behaviour of their trimethylsilyl derivatives on gas chromatography and in the case of MHPE confirmed this by mass spectral data. However, WILK (1971) was unable to detect MHPE in human cerebrospinal fluid. GOLDSTEIN, FRIEDHOFF, POMERANTZ and CONTRERA (1961) showed that DHPE and MHPE were metabolites of dopamine in the rat. BRAESTRUP (1972, 1973) has presented gas chromatographic evidence for the presence of conjugated MHPE in rat brain but has suggested that this is derived from the peripheral metabolism of dopamine. Dr. P. Ceasar and I have been unable to detect free MHPE in the stratum of the mouse even though we were able to measure the small amount of MHPG which is present in this tissue. The problem of the alcohol metabolites of dopamine in the cerebrospinal fluid requires further investigation.

THE REFLECTION OF NEURONAL ACTIVITY IN THE CONCENTRATION OF THE METABOLITES OF CATECHOLAMINES IN THE CEREBROSPINAL FLUID

The presence of metabolites of catecholamines in the brain and the cerebrospinal fluid poses the question whether the concentrations of such metabolites are related to the activity of the noradrenergic and dopaminergic neurons in the brain. With regard to the acidic metabolites it is unlikely that these are derived from the blood since the intravenous injection of HVA or 5HIAA, the acidic metabolite of 5-HT does not result in an increased concentration of these acids in the cerebrospinal fluid (ASHCROFT, DOW and MOIR, 1968; BARTHOLINI, PLETSCHER and TISSOT, 1966). The concentration of an acidic metabolite in cerebrospinal fluid appears to reflect the concentration of the metabolite in regions of the brain adjacent to the site of sampling when the two concentrations are allowed to come to equilibrium. MOIR, ASHCROFT, CRAWFORD, ECCLESTON and GULDBERG (1970) in reviewing this problem have pointed out that when the concentrations of HVA and 5HIAA in the caudate nucleus of the dog are increased after the administration of chlorpromazine the ratios of the concentrations of each of the acids in the caudate nucleus and the lateral ventricular cerebrospinal fluid remain constant (GULDBERG and YATES, 1969). The concentration

of 5HIAA in the cisternal fluid appears to reflect predominantly the concentration of 5HIAA in the brain stem region. BULAT and ZIVKOVIC (1971) have concluded that the 5-HIAA in the lumbar cerebrospinal fluid has its origin in the spinal cord. A similar origin for the MHPG in lumbar cerebrospinal fluid might be supposed since the spinal cord contains both 5-HT and noradrenaline. Although shown to be present, there is but very little dopamine in the rat spinal cord (ATACK, personal communication) and we have found only traces of HVA in rabbit spinal cord. It is thus unlikely that the greater part of the HVA in the lumbar cerebrospinal fluid is derived from this source. BULAT and ZIVKOVIC (1971) have shown that 5-HIAA, injected into the cisterna magna does not appear in the lumbar fluid although it disappears from the cisternal fluid. However, many samples of lumbar cerebrospinal fluid are taken from subjects which have had their life time to reach equilibrium and usually when more than one sample is taken a period of several days is allowed to elapse before a second sample is obtained. However, the observations of KORF, VAN PRAAG and SEBENS (1971) and of GORDON, OLIVER, GOODWIN, CHASE and POST (1973) show that when active transport of acids out of the cerebrospinal fluid is inhibited by probenecid then the concentrations of HVA and 5HIAA on the lumbar cerebrospinal fluid start to increase after four hours whereas the concentration of MHPG-sulphate is increased only after 18 hr. One further point, germane to this problem, is that, in the lateral ventricle, equilibrium between the acid metabolites in the tissue and the cerebrospinal fluid appears to be reached within one hour provided that small samples (0·5 ml) only are removed from the ventricle (ASHCROFT, DOW and MOIR, 1968).

The time course of a change in the activity of mono-aminergic neurons in the brain must be greatly attenuated in its reflection in a change in the concentration of the corresponding acidic metabolite in the cerebrospinal fluid. PORTIG and VOGT (1969) have shown that when the substantia nigra of the cat was stimulated electrically for four minutes there was an increased release of HVA into a perfusate of the lateral ventricle which could persist for more than one hour. This suggests that metabolites formed deep in the brain substance take a long time to reach the fluid in the ventricular system. There might be even greater difficulty in interpreting any changes which occur in concentrations of metabolites in the cisternal and lumbar cerebrospinal fluids since the acidic metabolites are known to be removed from the cerebrospinal fluid by a probenecid-sensitive, active transport system. This is thought to be situated in the choroid plexuses (ASHCROFT, DOW and MOIR, 1968; CSERR and VAN DYKE, 1971; PULLAR, 1971; FORN, 1972). The deciphering of the message contained on the concentrations of the metabolites of catecholamines in the cerebrospinal fluid is far from complete but the observations which have so far been made are such as to require that further research into this problem be carried out.

REFERENCES

ANDÉN N.-E., ROOS B.-E. and WERDINIUS B. (1963a) *Life Sci.* **2**, 448–458.
ANDÉN N.-E. ROOS B.-E. and WERDINIUS B. (1963b) *Experientia*, **19**, 359–360.
ARMSTRONG M. D., SHAW K. N. F. and WALL P. E. (1956) *J. biol. Chem.* **218**, 293–303.
ASHCROFT G. W., CRAWFORD T. B. B., DOW R. C. and GULDBERG H. C. (1968) *Br. J. Pharmac. Chemother.*, **33**, 441–456.
ASHCROFT G. W., DOW R. C. and MOIR A. T. B. (1968) *J. Physiol., Lond.*, **199**, 397–425.
ASHCROFT G. W. and SHARMAN D. F. (1960). *Nature, Lond.*, **186**, 1050–1051.
AXELROD J. (1966). *Pharmac. Rev.*, **18**, 95–113.

BARTHOLINI G., PLETSCHER A. and TISSOT R. (1966) *Experientia*, **22**, 609–610.
BOND P. A. (1972). *Biochem. Med.*, **6**, 36–45.
BRAESTRUP C. (1972). *Biochem. Pharmac.*, **21**, 1775–1776.
BRAESTRUP C. (1973). *J. Neurochem.*, **20**, 519–527.
BULAT M. and ŽIVKOVIĆ B. (1971) *Science*, **173**, 738–740.
CEASAR P. M. and SHARMAN D. F. (1972) *Br. J. Pharmac.*, **44**, 340–341P.
CORRODI H. and WERDINIUS B. (1965). *Acta chem. scand.*, **19**, 1854–1858.
CSERR H. F. and VAN DYKE D. H. (1971) *Amer. J. Physiol.*, **220**, 718–723.
DZIEDZIC S. W., BERTANI L. M., CLARKE D. D. and GITLOW S. E. (1972). *Analyt. Biochem.*, **47**, 592–600.
FORN J. (1972). *Biochem. Pharmac.*, **21**, 619–624.
GERBODE F. A. and BOWERS M. B. (1968) *J. Neurochem.*, **15**, 1053–1055.
GIBBS M. D. (1927) *J. biol. Chem.* **72**, 649–664.
GJESSING L. R., VELLAN E. J., WERDINIUS B. and CORRODI H. (1967). *Acta chem. scand.*, **21**, 820–821.
GOLDSTEIN M., FRIEDHOFF A. J., POMERANTZ S. and CONTRERA J. F. (1961) *J. Biol. Chem.*, **236**, 1816–1812.
GORDON E. K. and OLIVER J. (1971) *Clin. chim. Acta*, **35**, 145–150.
GORDON E. K., OLIVER J., GOODWIN F. K., CHASE T. N. and POST R. M. (1973). *Neuropharmacology*, **12**, 391–396.
GUILBAULT G. G., BRIGNAC P. J. Jr. and JUNEAU M. (1968). *Analyt. Chem.*, **40**, 1256–1263.
GULDBERG H. C. and YATES C. M. (1969) *Br. J. Pharmac.*, **36**, 535–548.
KIRSCHBERG C. J., COTE L. M., LOWE Y. H. and GINSBURG S. (1972) *J. Neurochem.*, **19**, 2873–2876.
KORF J., VAN PRAAG H. M. and SEBENS J. B. (1971) *Biochem. Pharmac.*, **20**, 659–668.
MANNARINO E., KIRSHNER N. and NASHOLD B. S. Jr. (1963) *J. Neurochem.*, **10**, 373–379.
MATHIEU P., CHARVET J. C., CHAZOT G. and TROUILLAS P. (1972) *Clin. Chim. Acta*, **41**, 5–17.
MATHIEU P., REVOL L. and TROUILLAS P. (1972) *J. Neurochem.*, **19**, 81–86.
MEEK J. L. and NEFF N. H. (1972). *Br. J. Pharmac.*, **45**, 435–441.
MOIR A. T. B., ASHCROFT G. W., CRAWFORD T. B. B., ECCLESTON D. and GULDBERG H. C. (1970) *Brain*, **93**, 357–368.
PORTIG P. J. and VOGT M. (1969). *J. Physiol., Lond.*, **204**, 687–715.
PULLAR I. A. (1971). *J. Physiol., Lond.*, **216**, 201–211.
RUTLEDGE C. O. and JONASON J. (1967) *J. Pharmac. exp. Ther.*, **157**, 493–502.
SATO T. L. (1965) *J. lab. clin. Med.*, **66**, 517–525.
SCHANBERG S. M., BREESE G. R., SCHILDKRAUT J. J., GORDON E. K. and KOPIN I. J. (1968) *Biochem. Pharmac.*, **17**, 2006–2008.
SCHANBERG S. M., SCHILDKRAUT J. J., BREESE G. R. and KOPIN I. J. (1968). *Biochem. Pharmac.*, **17**, 247–254.
SHARMAN D. F. (1960). The significance of pharmacologically active amines in animal tissues and body fluids. Ph.D. Thesis, University of Edinburgh.
SHARMAN D. F. (1963) *Br. J. Pharmac. Chemother.*, **20**, 204–213.
SHARMAN D. F. (1969) *Br. J. Pharmac. Chemother.*, **36**, 523–534.
SJÖQUIST B. and ÄNGGÅRD E. (1972) *Analyt. Chem.*, **44**, 2297–2301.
WATERBURY L. D. and PEARCE L. A. (1972) *Clin. Chem.* **18**, 258–262.
WILK S. (1971) *Biochem. Pharmac.*, **20**, 2095–2096.
WILK S., DAVIS K. L. and THACKER S. B. (1971). *Analyt. Biochem.*, **39**, 498–504.
WILK S., GITLOW S. E., CLARKE D. D. and PALEY D. H. (1967). *Clin. chim. Acta*, **16**, 403–408.