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UNEXPECTED OCCURRENCE OF PROLYLHYDROXYPROLINE DURING THE ANALYSIS OF COLLAGEN-BOUND CARBOHYDRATES BY GAS–LIQUID CHROMATOGRAPHY

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SUMMARY

Three unexpected peaks were observed in the gas–liquid chromatograms of trimethylsilylated samples of partially hydrolysed collagen. The most prominent peak was identified as being mainly the diketopiperazine of prolylhydroxyproline, and it is quantitatively reproducible.

INTRODUCTION

During the gas chromatographic analyses of collagen-bound carbohydrates[1,2] we observed three unexpected peaks which could not be identified as carbohydrate derivatives. As described below, it was finally concluded that the main unknown peak predominantly contained the trimethylsilylated (TMS) diketopiperazine of prolylhydroxyproline. The purpose of this paper is to warn other workers of this peak which is quantitatively reproducible.

EXPERIMENTAL

Hydrolysis

About 20 mg of air-dried material were hydrolysed in 3 ml of 1 *N* hydrochloric acid in a boiling water bath for 10 h, if not stated otherwise. The hydrolysates were lyophilised.

Gas chromatography

The silylation of the material was carried out by pipetting 0.3 ml of the silylation reagent, 1 ml of hexamethyldisilazane (purum, Fluka AG, Buchs SG, Switzerland) and 0.5 ml of trimethylchlorosilane (puriss., redistilled, Fluka) in 3.5 ml of pyridine, onto the dry samples and allowing to stand at room temperature for 30 min. Excess reagent was removed by evaporation in a stream of nitrogen at room temperature. The silylated derivatives were extracted with 3 ml of petroleum ether using ultrasonics to cause thorough mixing of the sample and ether, the solution was evaporated, and finally taken to 0.3 ml of petroleum ether. This step can be omitted and

the sample can be applied to the column in the silylation reagent. About 1–3 μl were injected into the gas chromatograph, except in the case of preparative fractionations, when 20 μl were used.

For analytical purposes columns of 6 ft. × 4 mm (Barber-Colman M-10) at 155° or 10 ft. × 3 mm (F & M 402) at 175° packed with 1% SE-30 on siliconised Gas-Chrom® P (Applied Science Laboratories, State College, Pa.) were used, if not stated otherwise. For preparative purposes, a 6 ft. × 10 mm column was used in the Barber-Colman apparatus together with a stream splitter (1:9). A flame ionisation detector and nitrogen as the carrier gas were used throughout.

Some experiments were carried out with the polar stationary phase 3% EGSS-X on Gas-Chrom® Q (Applied Science Laboratories) in 5 or 15 ft. × 3 mm columns (F & M 402) at 205° or 215°, respectively, More symmetrical peaks were obtained, but the instability of the chromatographic media inside the column was a disadvantage.

Determination of amino acid composition

The material was hydrolysed in 5.7 *N* hydrochloric acid under nitrogen at 110° for 20 h. Amino acid analysis was carried out by an automatic amino acid analyser built according to SPACKMAN *et al.*[3].

Radioactive labelling of materials in peaks X_1 and X_2

Rats weighing about 55 g were injected intraperitoneally with 130 μCi of either [^{3}H]proline or [^{3}H]lysine solutions (two rats in each group). After 24 h the animals were killed and the collagen of the tail tendons was extracted with 3% acetic acid for two days. The solubilised collagens were lyophilised, hydrolysed, trimethylsilylated and fractionated by gas chromatography on a preparative scale as described. The materials in peaks X_1 and X_2 (Fig. 1) were collected together in a capillary tube and then rinsed with 0.2 ml of methanol into the counting vial, which contained 10 ml of the scintillation fluid (15 g PPO and 50 mg POPOP in 1000 ml of toluene and diluted with methyl cellosolve in the ratio 10:6). The activity was counted with a Packard Tri-Carb Model 3214 scintillation spectrometer.

Infrared spectrometry

A Perkin-Elmer 257 grating infrared spectrometer was used. The material isolated by gas chromatography was trimethylsilylated again, dissolved in hexane, and allowed to evaporate on KBr, which was ground and compressed to a pellet.

Mass spectrometry

The combined LKB Model 9000 gas chromatograph–mass spectrometer was used either with a 6 ft. 2% SE-30 column at 230° and an ionisation energy of 70 eV for unknown material or with a 10 ft. 1% SE-30 column at 210° and ionisation energy of 20 eV for commercial L-prolyl-L-hydroxyproline (Cyclo Chemical Corporation, Los Angeles, Calif.).

RESULTS AND DISCUSSION

Occurrence of unexpected peaks

Fig. 1 shows a typical gas chromatogram. The retention times of peaks X_1, X_2

and X_3 with reference to α-glucose are 2.2, 2.9 and 4.4, respectively. The main emphasis of this work was placed on the principal component X_1.

These peaks were given by all the purified skin collagens investigated and obtained from several vertebrates[4], and by commercial gelatins (both alkali- and acid-treated) and by collagen fractions derived from the helical part of the tropocollagen molecule (obtained by pretreatment with pepsin). Peaks X_1, X_2 and X_3 were also found in urine although peak X_1 was very often split. They were not present in the

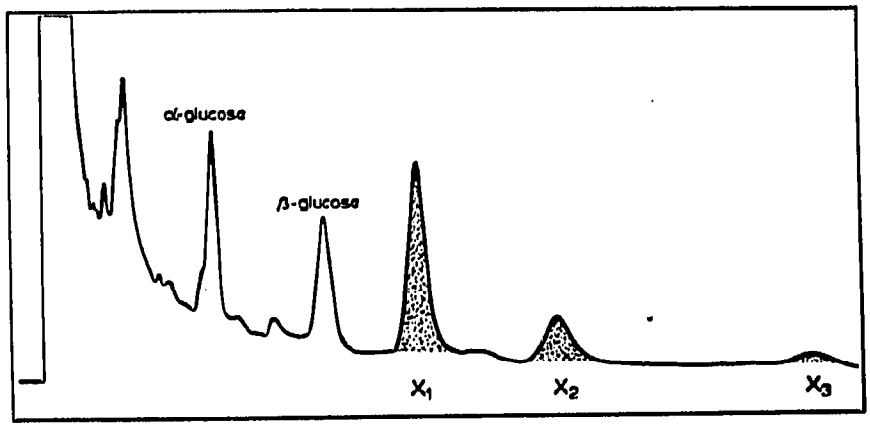

Fig. 1. Appearance of the unknown compounds X_1, X_2 and X_3 in a gas chromatogram of trimethylsilylated fragments of partially hydrolysed collagen. Hydrolysis in 1 *N* hydrochloric acid in a boiling water bath for 10 h. The α- and β-glucose peaks are derived from Sephadex®.

hydrolysates of Sephadex® or starch (used in the purification and the fractionation procedures) or in that of albumin. Neither hydroxylysine nor hydroxyproline alone yielded these peaks.

Incorporation of [^3H]proline and [^3H]lysine

In order to be sure that the peaks were derived from the tropocollagen molecule incorporation experiments were carried out as mentioned. The combined peaks X_1 and X_2 were found to be labelled (about 1600 d.p.m./20 mg of collagen) when [^{3}H]-proline had been injected. No incorporation of [^{3}H]lysine could be detected.

Amino acid composition of peak X_1 material

Preliminary experiments carried out by paper (first dimension: butanol–acetic acid–water, 60:15:25; second: phenol–1% ammonia) or thin-layer chromatography (first dimension: butanol–acetic acid–water, 60:15:25; second: chloroform–methanol–25% ammonia, 2:2:1) on the material from peak X_1 revealed some ninhydrin-positive spots. Their amount increased after total hydrolysis and the main spots were tentatively identified as proline, hydroxyproline, leucine and perhaps glycine. The results of the quantitative amino acid analyses are shown in Table I. The main component in peak X_1 seems to be a dipeptide composed of proline and hydroxyproline although a small amount of a dipeptide containing leucine and hydroxyproline is present in the second half of the peak, which is slightly asymmetrical (Figs. 1 and 4). A preliminary analysis of the material in peak X_2 revealed mainly serine and hydroxyproline.

TABLE I

AMINO ACID COMPOSITION OF THE MATERIAL IN PEAK X_1

The values have not been corrected for losses during the hydrolysis and chromatography.

Amino acid	*First half Residues/100*	*Second half Residues/100*
Hydroxyproline	36.3	38.5
Aspartic acid	0.8	1.1
Threonine	0.4	1.1
Serine	0.7	1.6
Glutamic acid	3.9	5.6
Proline	38.1	24.0
Glycine	5.1	5.6
Alanine	1.3	1.2
Valine	1.9	2.6
Methionine	0.4	1.8
Isoleucine	1.0	1.3
Leucine	7.3	12.8
Tyrosine	0.2	0.8
Phenylalanine	1.2	2.0
Ornithine	0.3	—
Lysine	1.1	—

Infrared spectroscopy of peak X_1 material

A comparison between the trimethylsilylated peak X_1 material and a commercial reference sample of prolylhydroxyproline supported the identification made on the basis of the amino acid analysis (Fig. 2).

Mass spectrometry

Comparison of the mass spectra representing the top of the gas chromatographic peak of X_1 material and commercial prolylhydroxyproline also confirms the results obtained by amino acid analysis (Fig. 3). The molecular weight 282 indicated by the peaks at *m/e* 282, 267 and 192 (M^+, M-15 and M-90)[5] is in agreement with the hypothesis that the dipeptide migrates in gas chromatography as the O-trimethylsilylated

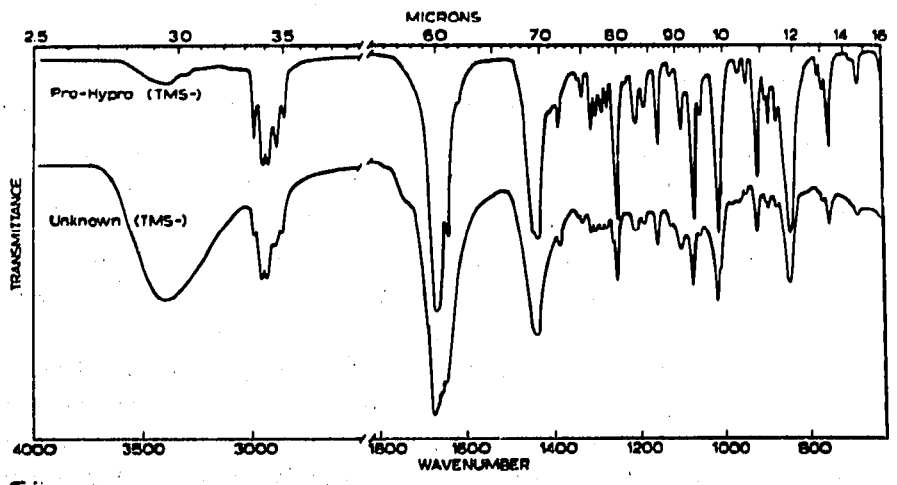

Fig. 2. Comparison between the infrared spectra of peak X_1 material (unknown) and L-prolyl-L-hydroxyproline.

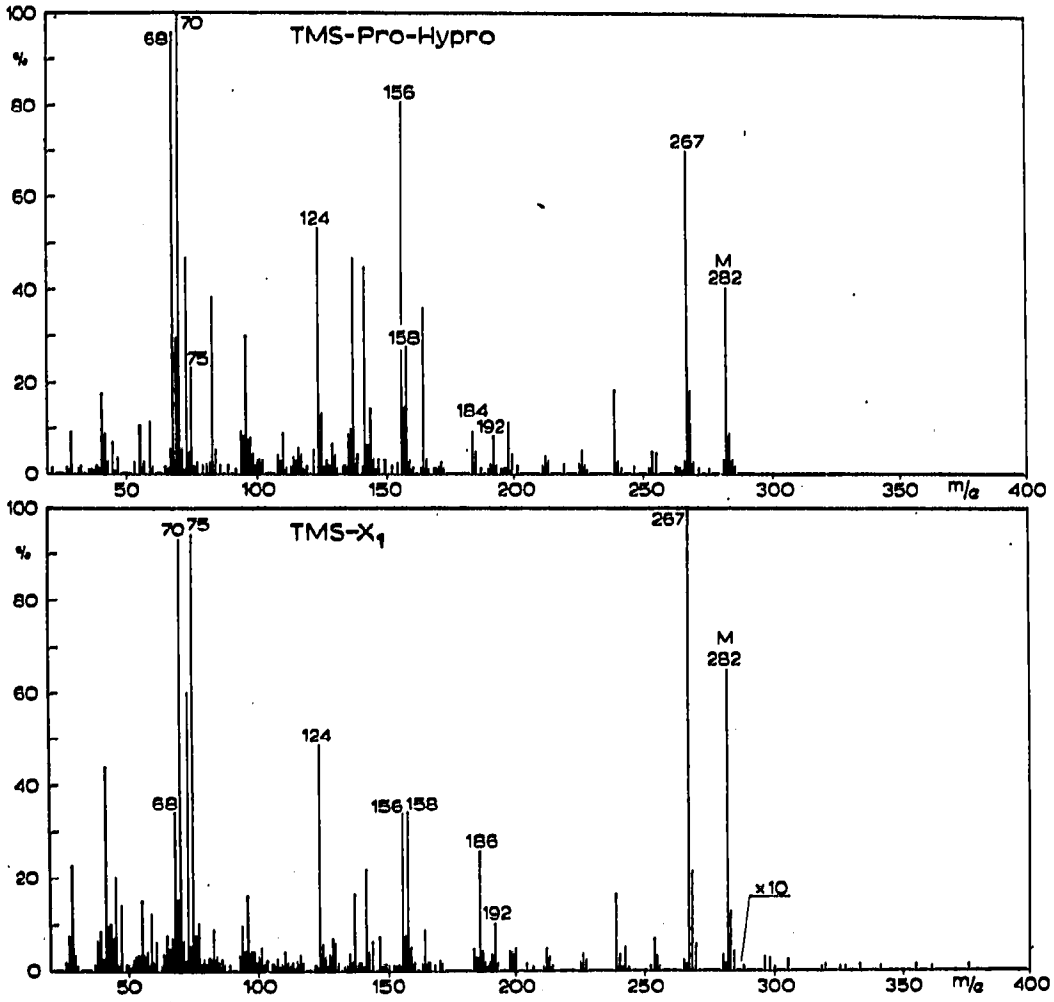

Fig. 3. Comparison between the mass spectra of material from peak X_1 (corresponding to the top of the GLC curve) and L-prolyl-L-hydroxyproline.

diketopiperazine. The ions at *m/e* 70 and 158 correspond to the "amine" fragments[6] arising from the prolyl and TMS-hydroxyprolyl moieties, respectively. Peaks two mass units lower (*m/e* 68 and 156), characteristic of the pyrrolidine ring, were also present and had high intensities. Of the remaining peaks, *m/e* 239 is thought to arise as a result of the elimination of CONH, and *m/e* 124 and 212 as a result of the elimination of the "amine" fragments of TMS-hydroxyproline and proline from the parent molecule. The ions at *m/e* 73 and 75 are most likely derived from the TMS-group[5]. The difference in the spectra can be partly explained by the use of two different mass spectrometers. The presence of some trimethylsilylated leucylhydroxyproline as diketopiperazine in peak X_1 could also be confirmed by mass spectrometry. Peak X_2 was tentatively identified as the diketopiperazine of di-O-TMS-serylhydroxyproline on the basis of its mass spectrum. This is in agreement with the amino acid analysis.

Presumably because of the rigid pyrrolidine ring the imino acid-containing dipeptides exhibit a marked tendency for intramolecular cyclisation, leading to the formation of diketopiperazines devoid of free amino or imino and carboxyl groups[7-9]. The absence of these polar groups is a condition for their gas chromatographic migration in the apolar SE-30 column used in this study.

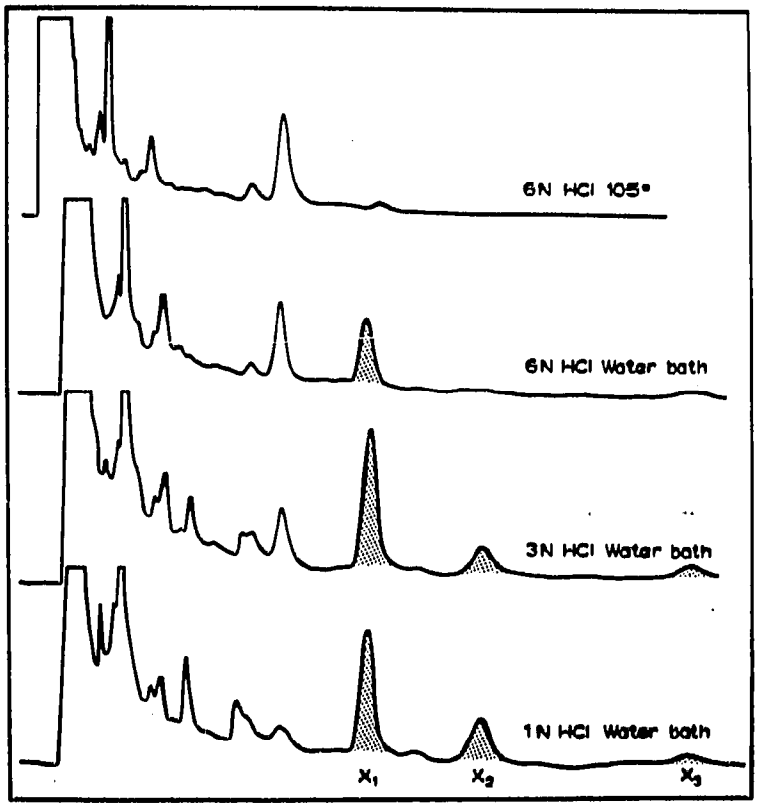

Fig. 4. Effect of the conditions during the hydrolysis on the appearance of unknown compounds in the gas chromatogram of trimethylsilylated fragments of collagen. Duration of hydrolysis 10 h.

At present the primary structure of the 135 amino acids from the amino terminal end of the α1-chain of rat skin collagen is known[10-12]. A third of the total hydroxyproline is included in the sequence Pro-Hypro. Glycine, in the helical part of the chain, always occupies position 1 in the triplet Gly-X-Y, whereas hydroxyproline is found in position 3 only. This predicts that the original sequences of the two dipeptides from peak X_1 are prolylhydroxyproline and leucylhydroxyproline, respectively.

The presence of glycylproline, as its N-trifluoroacetyl derivative, in a gas chromatogram of partially hydrolysed gelatin has been reported[13].

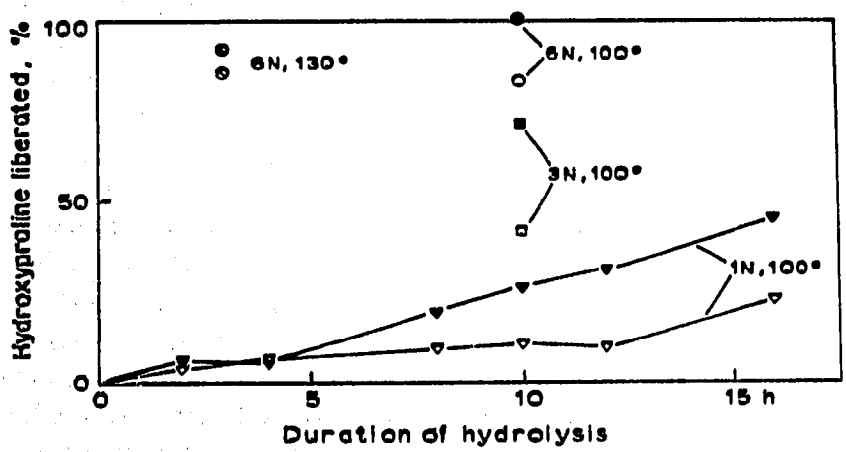

Fig. 5. Liberation of hydroxyproline from L-prolyl-L-hydroxyproline (open symbols) and from pig skin gelatin (closed symbols) in hydrochloric acid under conditions indicated.

Effect of hydrolysis conditions

An increase in the normality of the hydrochloric acid leads to the disappearance of these peaks. The effect of an increase in temperature is similar and more pronounced when the concentration of hydrochloric acid is higher (Fig. 4). Peaks X_2 and X_3 seem to be more sensitive than peak X_1. The hydrolysis time is, however, not critical when the hydrolysis is carried out in 1 *N* hydrochloric acid and in a boiling water bath.

The incompleteness of the hydrolysis of collagen or gelatin in 1 *N* hydrochloric acid at 100–105° for 10 h is obvious[14]. SCHROEDER *et al.*[15] claim that the peptide linkage in Pro-Hypro is weak. Our experience is different: in 1 *N* hydrochloric acid at 100° Pro-Hypro is broken very slowly (Fig. 5), about 1% per h. Pro-Hypro itself is liberated from gelatin under the same conditions during the first 4 h.

Eventual quantitative application

Using an internal standard, *e.g.* sorbitol, the analysis of prolylhydroxyproline can be made quantitative (Fig. 6), and so be used to determine the extent of the sequences Pro-Hypro or Hypro-Pro in the protein structure. When the hydroxylation of protocollagen proline is assessed, the resulting sequence Pro-Hypro can be deter-

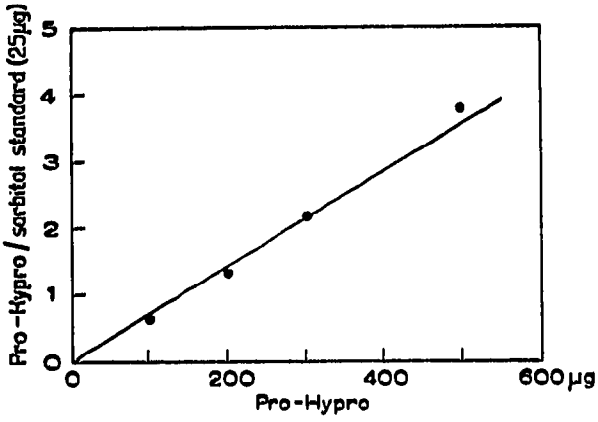

Fig. 6. Standard curve of L-prolyl-L-hydroxyproline with sorbitol as the internal standard added to the silylation mixture. The ordinate gives the ratios of the peak areas.

mined directly without radioactive compounds. In addition, Pro-Hypro is known to comprise about a half or two-thirds of the total hydroxyproline[16,17] in urine. The excretion of this dipeptide, which also is resistant to enzymic cleavage, represents the catabolism of collagen. However, for the determination of urinary Pro-Hypro, urea and salts have to be removed before silylation.

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REFERENCES

1 M. E. GRANT AND D. S. JACKSON, *Biochem. J.*, 108 (1968) 587.
2 T. RÖNNEMAA, J. PIKKARAINEN AND E. KULONEN, *Suom. Kemistil., B*, 44 (1971) 115.
3 D. H. SPACKMAN, W. H. STEIN AND S. MOORE, *Anal. Chem.*, 30 (1958) 1190.
4 J. PIKKARAINEN, *Acta Physiol. Scand.*, Suppl. 309 (1968) 1.
5 H. BUDZIKIEWICZ, C. DJERASSI AND D. H. WILLIAMS, *Mass Spectrometry of Organic Compounds*, Holden-Day, San Francisco, Calif., 1967.
6 H. J. SVEC AND G. A. JUNK, *J. Amer. Chem. Soc.*, 86 (1964) 2278.
7 H. D. DAKIN, *J. Biol. Chem.*, 44 (1920) 499.
8 E. L. SMITH AND M. BERGMANN, *J. Biol. Chem.*, 153 (1944) 627.
9 J. P. GREENSTEIN AND M. WINITZ, *Chemistry of the Amino Acids*, Vol. 3, John Wiley, New York, 1961, pp. 790, 1517, 1524.
10 A. H. KANG, P. BORNSTEIN AND K. A. PIEZ, *Biochemistry*, 6 (1967) 788.
11 P. BORNSTEIN, *Biochemistry*, 6 (1967) 3082.
12 W. T. BUTLER, in E. A. BALAZS (Editor), *Chemistry and Molecular Biology of the Intercellular Matrix*, Vol. 1, Academic Press, London, 1970, p. 149.
13 F. WEYGAND, B. KOLB, A. PROX, M. A. TILAK AND I. TOMIDA, *Z. Physiol. Chem.*, 322 (1960) 38.
14 R. L. HILL, *Adv. Protein Chem.*, 20 (1965) 37.
15 W. A. SCHROEDER, L. M. KAY, J. LEGETTE, L. HONNEN AND F. C. GREEN, *J. Amer. Chem. Soc.*, 76 (1954) 3556.
16 E. MEILMAN, M. M. URIVETZKY AND C. M. RAPOPORT, *J. Clin. Invest.*, 42 (1963) 40.
17 A. C. KIBRICK, H. L. POWER, E. SEVEDAL AND A. T. MILHORAT, *J. Clin. Endocrinol. Metab.*, 28 (1968) 1113.