The Separation of Ketosteroids by Partition Chromatography using Nitromethane as Stationary Phase

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Interest in the relationship of endocrine function to many diseases has led to much work upon the determination of the urinary ketosteroids. Estimation of the hydrolysed ketosteroid conjugates, after extraction and separation from phenolic oestrogens, is generally carried out by one of the many modifications of the Zimmermann reaction (reviewed by Nathanson & Wilson, 1943 and by Callow, 1950).

Investigations in this department (Bristol Mental Hospitals) have shown that the conclusions which can be drawn from the determination of the total ketosteroid excretion rate are very limited, since no qualitative or quantitative indication of the pattern of excreted ketosteroid is obtained. The systematic fractionation of urinary steroids into ketonic and non-ketonic portions by means of Girard's reagent T (Girard & Sandulesco, 1936), and into 3α- and 3β-hydroxysteroids by the use of digitonin (Frame, 1944) has provided more useful information. However, the production of artifacts, e.g. 3β-chloroandrost-5-en-17-one, during the initial hydrolysis can lead to errors in interpretation of the results of the digitonin precipitation.

Adsorption chromatography has been applied to the separation of excreted ketosteroids, and many new substances have been identified recently by means of this technique (Lieberman, Dobriner, Hill, Fieser & Rhoads, 1948; Lieberman, Fukushima & Dobriner, 1950). Many ketosteroid fractionations have been carried out upon alumina columns by Dingemanse, Huis in't Veld & Laat (1946) and by Dingemanse, Huis in't Veld & Hartogh-Katz (1952).

Interest in the separation of compounds that are sparingly soluble in water, by means of partition chromatography with a non-aqueous stationary phase, led us to consider the use of this procedure for the separation of ketosteroid mixtures.

Boscott (1947) suggested the use of cellulose acetate for phase reversal and Boldingh (1948, 1950) successfully separated the methyl esters of some fatty acids upon rubber-impregnated paper partition chromatograms and upon columns of rubber powder. The successful use of reversed phase chromatography for the separation of fatty acids upon columns of kieselguhr treated with dichlorodimethylsilane has been described by Howard & Martin (1950). Ramsey & Patterson (1946, 1948) were able to separate the components of commercial hexachloro*cyclo*hexane and the higher fatty acids upon columns of silica gel which held a stationary polar organic liquid (nitromethane or methanol).

Counter-current extraction methods have been applied by Engel, Slaunwhite, Carter & Nathanson (1950), for resolving the natural oestrogens, and paper partition chromatography has been applied fairly widely for the analysis of steroids; systems have been described by Kritchevsky & Calvin (1950) for resolving mixtures of cholesterol and cholestenone. Heftmann (1951) separated oestradiol-17α from oestradiol-17β by partition chromatography upon paper, and Zaffarroni, Burton & Keutmann (1950, 1951) achieved valuable separations of the corticosteroids upon papers containing formamide or propylene glycol as stationary phase. A method described for the separation of ketosteroids by partition chromatography (Zaffaroni, Burton & Keutmann, 1949) depended upon the formation of water-soluble hydrazones and the use of papers with aqueous stationary solvent systems. Kritchevsky & Tiselius (1951) have described the separation of free ketosteroids upon silicone-treated papers with an aqueous ethanol/chloroform solvent, and Kritschevsky & Kirk (1952) have recently described the separation of steroids on papers treated with 'Quilon (steratochromic chloride)'.

We have used filter papers impregnated with alumina (Stitch, 1951) for the qualitative separation of ketosteroids. Efforts to make this method quantitative, by extraction of the separated steroids from the paper and subsequent colorimetric estimation, were unsatisfactory because of high blanks. It was not found possible to reduce these blanks to satisfactory limits by preliminary purification of the papers by washing with sodium hydroxide or by organic solvent extraction. Attempts to run paper partition chromatograms with acetic anhydride (cf. Moynihan & O'Colla, 1951) or vaseline (cf. Winteringham, Harrison & Bridges, 1950) as stationary phase for the separation of corticosteroids were unsatisfactory.

Rubber-powder partition columns have been used by Nyc, Maron, Garst & Friedgood (1951) for the separation of the natural oestrogens, and the separation of some steroids upon Super-Cel columns with aqueous methanol as stationary phase has been described by Butt, Morris, Morris & Williams (1951).

We have investigated several potential methods for the separation of ketosteroids upon columns by partition chromatography, including a synthetic rubber powder Alloprene B (Imperial Chemical Industries Ltd. Southern Division) and Mealorub rubber powder (Rubber-stichting, Delft, Netherlands), cellulose acetate and benzyl cellulose. The slight solubility of these substances in the solvents employed and the lack of suitable solvent pairs rendered these materials unsuitable for our purposes.

However, we found that small silicic acid columns, similar to those employed by Ramsey & Patterson (1946) and containing nitromethane as stationary phase, provide an efficient means for the separation of some ketosteroid mixtures and for the fractionation of urinary ketosteroid extracts.

EXPERIMENTAL METHODS

Reagents

Ethanol. This was purified for the Zimmermann reaction according to the method described by Reiss, Hemphill, Gordon & Cook (1949).

Light petroleum. The A.R. grade, boiling range 60–80°, or A.R. *n*-hexane, was redistilled through a six-pear column.

Chloroform and benzene. The A.R. grades were redistilled through six-pear columns.

Nitromethane. This reagent (British Drug Houses Ltd.) was redistilled under reduced pressure.

Silicic acid. Silicic acid moist (British Drug Houses Ltd.) was dried in shallow dishes at a temperature not exceeding 50° during 24 hr. or until the material fell to a dry white powder which was then ground with pestle and mortar and sieved. The dry silicic acid passing 100-mesh was purified by continuous extraction with ether in a Sohxlet apparatus for approx. 6 hr. This procedure removed ether-soluble impurities and ensured very low blanks during subsequent colorimetric evaluation of the column eluates. The purified and dried silicic acid powder was stored in air-tight bottles, or in a desiccator.

Columns

Experiments with a large number of columns have revealed that effective separations of many ketosteroid mixtures may be achieved rapidly upon short columns of silicic acid containing nitromethane, although separation of the more closely related substances, such as androstan-3β-ol-17-one and androst-5-en-3β-ol-17-one, was best achieved upon the longer columns described below.

Pyrex-glass columns 20 × 300 mm. and fitted with glass taps have been used. The purified silicic acid powder (10 g.) was stirred in a beaker with the slow addition of nitromethane (5 ml.), the powder retaining its dry appearance at this stage. The mobile phase (100 ml. approx.), consisting of 3% (v/v) $CHCl_3$ in light petroleum and saturated with nitromethane, was added. Trapped bubbles were removed by thoroughly stirring the slurry with a glass rod. After placing a small cotton-wool plug in the bottom of the column, the slurry was poured in and washed down with additional small quantities of the eluent. The column was then compressed quickly under a pressure of N_2 or air of about 4 lb./sq.in., or allowed to settle under gravity for 2 or 3 hr. until a convenient flow rate of about 1–1·5 ml./min. was obtained. In order to standardize the threshold volumes of the steroids, the columns were allowed to run usually for not more than 1 hr. before loading; longer running may increase the amount of nitromethane initially present as stationary phase. No advantage appears to be obtained when these columns are run more slowly.

The band from the dye Sudan III (red) could be used, if desired, in order to test the column for even packing; a perfectly horizontal and sharp band should descend the column when developed with the mobile phase described above. This test, however, is not essential as it appears that an evenly packed column can be obtained if all trapped bubbles are removed by stirring and the column is not allowed to become dry.

Loading the column

The steroid mixture for analysis must be introduced carefully to prevent disturbance of the gel surface. Loading was best achieved by means of a glass pipette drawn out to a fine point (approx. 0·5 mm. diameter) and fitted with a rubber teat. The glass tap at the bottom of the column was opened and the mobile liquid allowed to disappear into the surface of the silica gel. The steroid mixture, dissolved in the minimum quantity of eluent, was then applied to the top of the column by means of the pipette. When the steroid solution had passed into the surface of the column, it was washed in with three successive small portions (2 ml.) of the mobile solvent, before placing a reservoir of this liquid in position. Effluent 5 ml. fractions were collected by means of an automatic receiver changer (Grant & Stitch, 1951).

Colorimetric estimation of the fractionated ketosteroids

The fractions from the column were transferred to 150 × 15 mm. Pyrex test tubes and evaporated to dryness at a temperature not exceeding 100°. Traces of nitromethane, which would interfere with subsequent colorimetric analysis, were removed under reduced pressure from a water pump. The Zimmermann reaction, as modified by Callow, Callow & Emmens (1938), was carried out, and the ketosteroid content of each fraction determined from a calibration curve in terms of androst-5-en-3β-ol-17-one. It has been shown by Callow *et al.* (1938) that the Zimmermann reaction produces, with different 17-ketosteroids, similar colour intensities per mg. of material.

The position of the separated steroids was revealed as a series of peaks upon graphs in which the ketosteroid concentration was plotted against the volume of eluate or fraction number.

Preparation of urinary extracts for chromatography

The 24 hr. urine samples have been hydrolysed and extracted according to two methods; some were subjected to the simultaneous acid hydrolysis and CCl_4 extractions

described by Callow *et al.* (1938) and some to the neutral benzene and acid benzene extractions described by Dingemanse *et al.* (1952).

After hydrolysis, the crude concentrated solvent extracts were washed with 2×50 ml. of water, 3×50 ml. of 2N-NaOH and 3×50 ml. of water, dried with Na_2SO_4 overnight, and, after concentration and filtration, made up to 50 ml. in a volumetric flask. Estimation of the crude total ketosteroid was then carried out by means of the Zimmermann reaction, and a portion of the crude total ketosteroid solution containing material equivalent to 5 mg. of androst-5-en-3β-ol-17-one taken and separated into ketonic and non-ketonic fractions by means of Girard's reagent T. The total ketonic material was then similarly estimated by means of the Zimmermann reaction, and the whole of this solution, or a portion not exceeding 3·5 mg., taken for chromatography. This solution of ketonic steroids was taken to dryness in a Pyrex test tube, 0·2 ml. of purified ethanol added in order to ensure complete solution of the material and the mobile phase (5 ml.), previously described, added and the solution loaded on the chromatogram. Fractions of the eluate collected by means of the automatic fraction collector, were, after evaporation of solvent, estimated by means of the Zimmermann reaction.

RESULTS

Pure steroids

The behaviour of individual steroids upon the partition chromatograms has been studied. Each pure substance tested was eluted at a characteristic position in the elution sequence and gave rise to a single band on the chromatogram. Examples are

Table 1. *Threshold volumes of some ketosteroids upon silicic acid columns*

(Conditions as described on p. 680; 10 g. silicic acid.)

Substance	Threshold vol. (ml. approx.)
Cholest-4-en-3-one	24
3β-Chloroandrost-5-en-17-one	33
3:5-*cyclo*Androstan-6-ol-17-one	90
Androsterone	90
Androstan-3β-ol-17-one	130
Androst-5-en-3β-ol-17-one	170
Testan-3α-ol-17-one	210
Testane-3α:11β-diol-17-one	210
Androst-4-en-3:17-dione	270

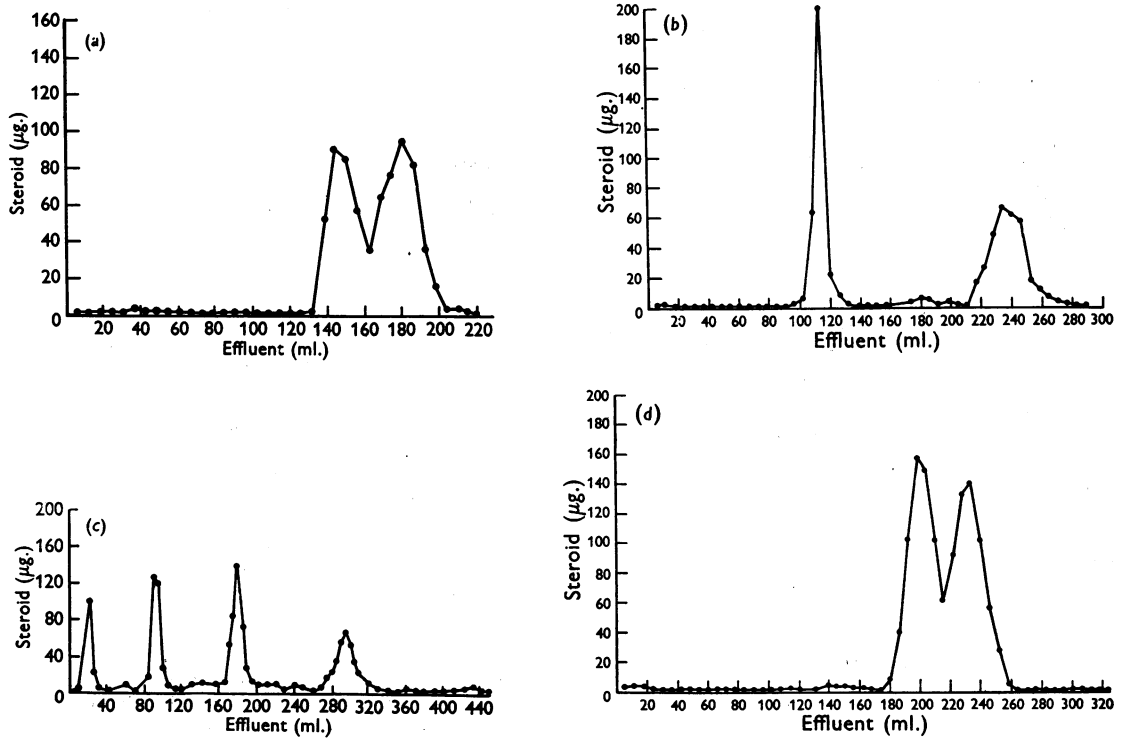

Fig. 1. Separation of some steroid mixtures upon silicic acid columns (steroid mixture (1–2 mg. approx.) on 10 g. SiO_2 column. The ordinates in this and other figures marked Steroid (μg.) indicate the quantities of chromogenic material as their equivalent in μg. of androst-5-en-3β-ol-17-one. (*a*) Androst-5-en-3β-ol-17-one (dehydro*epi*androsterone) and androstan-3β-ol-17-one (*epi*androsterone). (*b*) Androstan-3α-ol-17-one (androsterone) and testan-3α-ol-17-one (aetiocholanolone). (*c*) Cholest-4-en-3-one, androsterone, androst-5-en-3β-ol-17-one and androst-4-ene-3:17-dione. (*d*) Androst-5-en-3β-ol-17-one and testan-3α-ol-17-one (aetiocholanolone).

illustrated in Fig. 2*a*. The threshold volumes for some ketosteroids upon the partition chromatogram are given in Table 1.

The separation of mixtures of pure steroids has been studied and some typical results are shown in Fig. 1.

The conditions described above (10 g. silicic acid columns with a mobile solvent containing 3% of chloroform) are convenient but arbitrary; when followed rigidly they produce rapid separations in

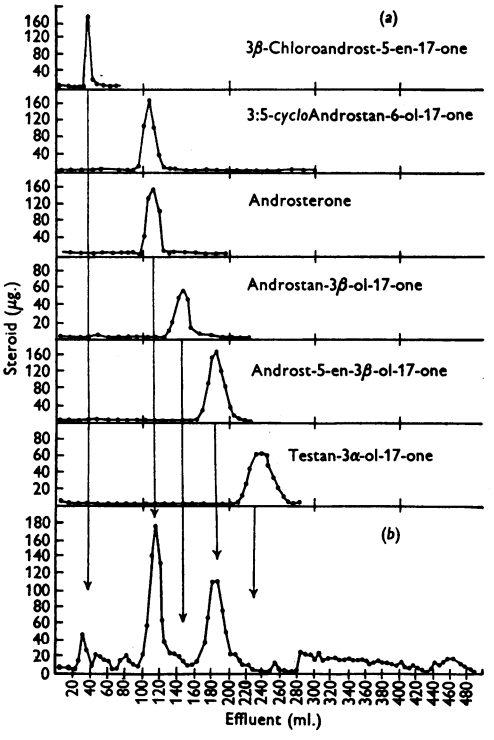

Fig. 2. (*a*) Behaviour of individual ketosteroids upon silicic acid columns (10 g.). (*b*) Pattern obtained from the urinary ketosteroid extract of a normal female (age, 26 years). Hydrolysis and extraction carried out according to the method of Dingemanse *et al.* (1952).

which the sequence and threshold volumes of the steroids are constant. Adequate separations have been obtained upon shorter columns holding 8 g. of material. A fractionation such as that shown in Fig. 2*b* can conveniently be obtained within 4 hr. on a 10 g. column.

Urine extracts

Chromatograms of three urine extracts are shown in Figs. 2 and 3. The positions occupied by known steroids in the urine extract in Fig. 2*b* can be seen from Fig. 2*a*. Some of the peaks obtained in the extract may not be composed solely by that steroid indicated, since the small amount of material, namely 2·5–3·5 mg., used for the fractionation of an urinary ketosteroid extract has so far precluded the characterization of the peaks from a single column. The positions of androstane-3α:11α-diol-17-one, and testan-3α-ol-11:17-dione in the elution sequence have yet to be ascertained, since these substances have not been available to us. It is to be expected, however, that they will be eluted towards the end of the sequence, since it can be seen from Table 1 that the threshold volume of the steroids increases with the polarity of the molecule.

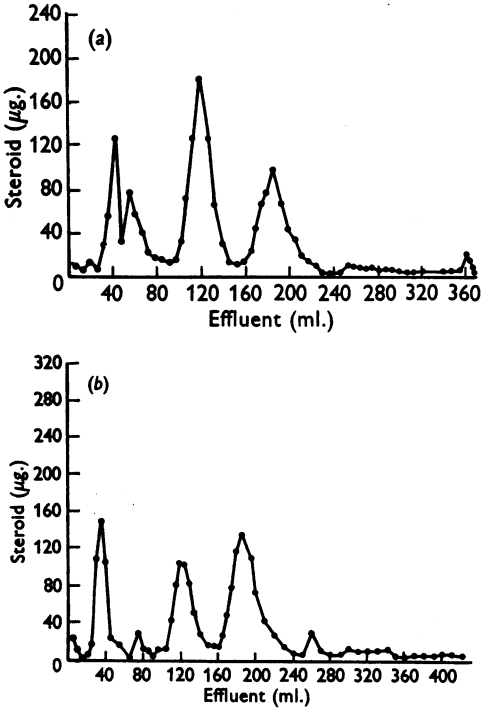

Fig. 3. (*a*) Pattern obtained from the urinary ketosteroid extract of a normal male, age 24 years. Hydrolysis and extraction carried out according to the method of Dingemanse *et al.* (1952). (*b*) Pattern obtained from the urinary ketosteroid extract of a male hospital patient age 38 years, an inadequate psychopath with schizophrenic and depressive features. Hydrolysis and extraction carried out by the method of Callow *et al.* (1938).

Artifacts obtained during extraction

The production of artifacts during extraction of ketosteroids from urine is well known. We have refluxed pure androsterone in an acid carbon tetrachloride/water system, simulating the conditions of the extraction method of Callow *et al.* (1938). Chromatography of the extract revealed a pattern as shown in Fig. 4*a*. When androst-5-en-3β-ol-17-one was refluxed under similar conditions, three peaks were obtained, one of which corresponded to

the position of 3β-chloroandrost-5-en-17-one (see Fig. 4*a*).

It has been suggested by Mason & Engstrom (1950) that 3:5-*cyclo*androstan-6-ol-17-one (formerly called *i*-androstenolone), may be produced from the

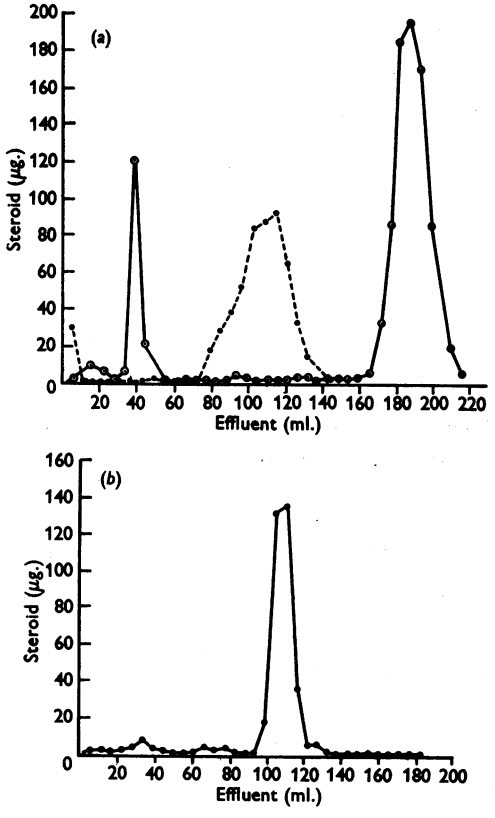

Fig. 4. (*a*) Chromatographic patterns obtained after steroids had been subjected to the acid extraction conditions of Callow *et al.* (1938). ——, Androst-5-en-3β-ol-17-one; - - - -, androsterone. (*b*) Chromatographic pattern obtained after the pyridinium salt androst-5-en-3β-ol-17-one sulphate had been subjected to the neutral benzene extraction conditions of Dingemanse *et al.* (1952).

sulphate of androst-5-en-3β-ol-17-one by the action of boiling benzene. Liebermann, Engel & Davies (1952: private communication through Dr R. I. Dorfman), have obtained 3:5-*cyclo*androstan-6-ol-17-one in good yield by refluxing the above sulphate with benzene.

We have refluxed the pyridinium salt of androst-5-en-3β-ol-17-one sulphate in a neutral benzene/water system similar to that employed for the neutral extraction of urine (Dingemanse *et al.* 1952). Chromatography of the extract gave rise to a peak in the position corresponding to 3:5-*cyclo*androstan-6-ol-17-one (Fig. 4*b*). Lengthy neutral extractions of urine with benzene therefore appear undesirable on a routine practice.

These results indicate the changes that steroids may undergo during extraction, but take no account of the possible influence of other substances present in urine upon these changes. The results emphasize that caution is necessary in the interpretation of the results of ketosteroid fractionation.

SUMMARY

1. A rapid method is described for the separation of some ketosteroid mixtures by partition chromatography upon columns of silicic acid containing nitromethane as stationary phase and a light petroleum/chloroform mixture, saturated with nitromethane, as the mobile phase.

2. Some urinary ketosteroid extracts have been fractionated in order to illustrate the utility of the method.

3. Some observations on the production of ketosteroid artifacts during the neutral and acid extractions of urine are discussed.

4. The method permits the separation of closely related steroid pairs such as androst-5-en-3β-ol-17-one and androstan-3β-ol-17-one and of androsterone (androstan-3α-ol-17-one) and testan-3α-ol-17-one.

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REFERENCES

Boldingh, J. (1948). *Experientia*, **4**, 270.
Boldingh, J. (1950). *Rec. Trav. chim. Pays-Bas*, **69**, 247.
Boscott, R. J. (1947). *Nature, Lond.*, **159**, 342.
Butt, W. R., Morris, P., Morris, C. J. O. R. & Williams, D. C. (1951). *Biochem. J.* **49**, 434.
Callow, R. K. (1950). In *Hormone Assay*, ed. by C. W. Emmens. New York: Academic Press Inc.
Callow, N. H., Callow, R. K. & Emmens, C. W. (1938). *Biochem. J.* **32**, 1312.
Dingemanse, E., Huis in't Veld, L. G. & Hartogh-Katz, S. L. (1952). *J. clin. Endocrin.* **12**, 66.
Dingemanse, E., Huis in't Veld, L. G. & Laat, B. M. de (1946). *J. clin. Endocrin.* **6**, 535.
Engel, L. L., Slaunwhite, W. R., Carter, P. & Nathanson, I. T. (1950). *J. biol. Chem.* **185**, 255.

Frame, E. (1944). *Endocrinology*, **34**, 175.
Girard, A. & Sandulesco, G. (1936). *Helv. chim. acta*, **19**, 1095.
Grant, R. A. & Stitch, S. R. (1951). *Chem. & Ind.* (Rev.), p. 230.
Heftmann, E. (1951). *J. Amer. chem. Soc.* **73**, 851.
Howard, G. A. & Martin, A. J. P. (1950). *Biochem. J.* **46**, 532.
Kritchevsky, D. & Calvin, M. (1950). *J. Amer. chem. Soc.* **72**, 4330.
Kritchevsky, D. & Kirk, M. R. (1952). *J. Amer. chem. Soc.* **74**, 4484.
Kritchevsky, T. H. & Tiselius, A. (1951). *Science*, **114**, 299.
Lieberman, S., Dobriner, K., Hill, B. R., Fieser, L. F. & Rhoads, C. P. (1948). *J. biol. Chem.* **172**, 263.
Lieberman, S., Fukushima, D. L. & Dobriner, K. (1950). *J. biol. Chem.* **182**, 299.
Mason, H. L. & Engstrom, W. W. (1950). *Physiol. Rev.* **30**, 321.
Moynihan, P. & O'Colla, P. (1951). *Chem. & Ind.* (Rev.), p. 407.
Nathanson, I. T. & Wilson, H. (1943). *Endocrinology*, **33**, 189.
Nyc, J. F., Maron, D. M., Garst, J. B. & Friedgood, H. B. (1951). *Proc. Soc. exp. Biol., N.Y.*, **77**, 466.
Ramsey, L. L. & Patterson, W. I. (1946). *J. Ass. off. agric. Chem. Wash.* **29**, 337.
Ramsey, L. L. & Patterson, W. I. (1948). *J. Ass. off. agric. Chem. Wash.* **31**, 441.
Reiss, M., Hemphill, R. E., Gordon, J. J. & Cook, E. R. (1949). *Biochem. J.* **44**, 632.
Stitch, S. R. (1951). *J. Endocrin.* **7**, xvii.
Winteringham, F. P. W., Harrison, A. & Bridges, R. G. (1950). *Nature, Lond.*, **166**, 999.
Zaffaroni, A., Burton, R. B. & Keutmann, E. H. (1949). *J. biol. Chem.* **177**, 109.
Zaffaroni, A., Burton, R. B. & Keutmann, E. H. (1950). *Science*, **111**, 6.
Zaffaroni, A., Burton, R. B. & Keutmann, E. H. (1951). *J. biol. Chem.* **188**, 763.

Role of Acetoacetate in Aggravating the Disturbances in the Carbohydrate Metabolism of Scorbutic Animals

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Depletion of vitamin C has been reported by Banerjee & Ghosh (1947) to disturb carbohydrate metabolism. It has also been shown by Attenburger (1936), Shimamura (1938) and Amorelli & D'Ambrosio (1938) that vitamin C is necessary for glycogenesis and that its deficiency in the system brings about a marked depletion in glycogen content of the liver as well as of the muscle. The recent findings of Nath & Chakrabarti (1951) in this laboratory have indicated that repeated injections of acetoacetate cause a marked depletion in the blood vitamin C level of rabbits. The present work was, therefore, undertaken by the authors to study the changes in carbohydrate metabolism following acetoacetate injections in their animals. The present paper describes the increase in blood sugar and rapid depletion in glycogen storage of guinea pigs which were completely denied vitamin C in their diet and received daily acetoacetate in gradually increased amounts. The effect on vitamin C excretion of guinea pigs under different experimental conditions was also studied.

EXPERIMENTAL

A group of forty-eight normal male guinea pigs with an average weight of 464 g. was selected for the pre-experimental period. Each one of them was given green grass and germinated gram in the morning and the scurvy-producing diet of Banerjee (1945) with slight modification (wheat being substituted for barley) in the evening, and this was continued for 3 days. From the fourth day the green grass and germinated gram, the sources of vitamin C, were discontinued and all the guinea pigs were fed *ad lib.* the scurvy-producing diet for 1 week, supplemented daily with 5 mg. of ascorbic acid given orally, and every third day two drops of a concentrated solution of vitamins A and D (Adexolin; Glaxo Laboratories) were given to each. The weights of the animals were recorded every third day and only those which were found to be gaining in weight were finally selected for the experimental period. The animals thus selected were divided into different groups in such a manner that the average weight of each group was nearly the same. The first group served as control and was kept on the basal diet with a daily oral supplement of 5 mg. ascorbic acid per day to each. The second group was denied vitamin C and was given only the scurvy-producing diet. The animals in the third group were also completely denied vitamin C, but received daily acetoacetate in increasing amounts (175 mg./kg./day given intraperitoneally and increased by 40 mg. every third day so that at the end of the experimental period of 21 days the dose was 415 mg./kg.). All the above three groups were further divided into sub-groups *A* and *B* for the observation of blood-sugar values and glycogen storage, respectively. To the fourth group, denied vitamin C, glucose was given daily in a constant dose of 400 mg./kg. (intraperitoneally).

Effect on blood sugar following acetoacetate injections in increasing amounts to guinea pigs completely denied vitamin C