Table 15. *Rate of excretion of ingested nicotinic acid*

Time	Nicotinic acid excreted (mg./hr.)
3 hr. period before dosing	0.23
Total 24 hr. period before dosing	1.26
100 mg. nicotinic acid given by mouth	
0 hr.	—
1	7.77
2	2.46
3	0.66
4	0.37
5	0.25
5 hr. total excretion	11.51
5-24 hr.	2.18
24 hr. total excretion	13.69

increase in the excretion of trigonelline and related N-methyl pyridinium compounds which can be regarded as normal metabolites. The fate of ingested nicotinic acid or amide in the body has been briefly discussed in our previous report [Kodicek & Wang, 1941]. The detailed results will be published in a subsequent paper. On the basis of these findings, we have recommended dosing with nicotinic amide, followed by the estimation of the urinary excretion both of trigonelline and nicotinic acid, as a method of assessing the level of nutrition with regard to the anti-pellagra vitamin.

SUMMARY

1. The cyanogen bromide-*p*-aminoacetophenone method of Harris & Raymond for the estimation of

nicotinic acid in urine has been further studied, and several additional improvements have been introduced, viz. (a) decolorization of the digested urine by washing with isobutanol and oxidation with KMnO_4 ; (b) the use of a lower temperature, a shorter time for the reaction of CNBr with nicotinic acid, and the addition of larger amounts of *p*-aminoacetophenone to the reaction mixture; and (c) the introduction of a modified blank.

2. The normal excretion of nicotinic acid by ten healthy subjects and the response to test doses of nicotinic acid by four of them has been studied.

3. The maximum excretion of ingested nicotinic acid occurs in the first three hours, the first hour showing the highest output.

4. It is emphasized that when test doses of nicotinic acid are used for assessing the body reserves of the vitamin, they should be administered under standardized conditions.

5. For obtaining reliable information of the nutritional status in regard to the anti-pellagra vitamin, dosing with nicotinic amide is to be preferred to dosing with nicotinic acid, and estimation should then be made of the excretion of trigonelline, as well as that of other substances related to nicotinic acid.

We wish to take this opportunity to thank Dr L. J. Harris for his constant interest and valuable advice; Mr G. G. Grindley for volunteering as an experimental subject, and Roche Products Ltd. for a gift of *p*-aminoacetophenone.

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The Etherification of Hydroxyamino-acid Residues in Silk Fibroin by Dimethyl Sulphate

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(Received 29 July 1943)

In recent years it has been recognized that in addition to tyrosine the hydroxyamino-acids serine and threonine are major protein constituents. The mode

of linkage of the —OH groups of these latter in the protein is of interest, since such groups might play a part in non-peptide linkage of amino-acid residues

[Chibnall, 1942; Linderstrøm-Lang & Jacobsen, 1941], or, if free, influence significantly the chemical and physical properties of the protein. Colour reactions and spectroscopy have thrown considerable light on the mode of incorporation in proteins of the phenolic —OH groups of tyrosine, but these methods are not of value in studying aliphatic —OH groups. The acylation of proteins has been studied by numerous workers, but, while the number and lability of acyl groups introduced provides circumstantial evidence for the occurrence of free —OH groups, the lability of *O*-acyl groups to the usual hydrolytic agents has precluded confirmation of this by the isolation of *O*-acylated compounds from hydrolysates of acylated proteins.

Methylation has been extensively and successfully employed for investigating the mode of linkage of aliphatic —OH groups in carbohydrates. Many workers, for a variety of purposes, have applied methylating agents to proteins [Blackburn, Carter & Phillips, 1941], but few [e.g. Skraup & Krause, 1909; Haurowitz, 1938] have employed conditions suitable for methylating aliphatic —OH groups, and of these none seems to have identified *O*-methylated compounds after hydrolyzing the protein. The strongly alkaline aqueous conditions, which appear necessary for the methylation of aliphatic —OH groups in proteins, induce degradative changes in the protein, making interpretation more complicated. Nevertheless, it seemed worth attempting the application of this technique in the protein field.

We have studied the action of dimethyl sulphate on silk fibroin in the presence of excess cold aqueous NaOH. This protein was chosen because of its resistance to solution by cold alkaline reagents, which facilitates repeated treatment, its negligible S content and its high content of hydroxyamino-acids.

Repeated treatment of silk fibroin in this way introduces *O*-methyl groups to an extent which cannot be increased by continued treatment. At this stage nearly all the tyrosine residues and about one-half the serine residues have been converted to their *O*-methyl derivatives. Our evidence for this is: (1) Disappearance of those colour reactions (Millon, xanthoproteic) due to free —OH groups of tyrosine residues from methylated fibroin and its hydrolysates. (2) Diminution of the hydroxyamino-acid content (NH_3 liberated by periodate) of hydrolysates of methylated fibroin to about one-half of the corresponding value for normal fibroin. (*O*-methyl-serine does not give NH_3 with periodate.) (3) The —OMe content of methylated fibroin and its hydrolysates corresponds stoichiometrically to the tyrosine + half the hydroxyamino-acid content of fibroin. (4) The occurrence of *O*-methyl-tyrosine and *O*-methyl-serine in hydrolysates of methylated fibroin. These have been demonstrated in the ex-

pected quantities, after conversion to their *N*-acetyl derivatives. For the latter compound we have employed partition chromatography on silica gel [cf. Gordon, Martin & Synge, 1943*a*].

Preliminary acetylation of the fibroin by boiling acetic anhydride [cf. Carr, 1938*b*] was found not to increase the amount or rapidity of methylation. Preliminary acetylation has been found useful in the methylation of polysaccharides [cf. Hirst & Young, 1939].

It was found that fully methylated fibroin on subsequent acetylation took up correspondingly fewer *O*-acetyl groups (determined by saponification with cold alkali) than normal fibroin. However, some acetyl groups saponifiable under these conditions were obtained on acetylation of fully methylated fibroin.

From the above it is apparent that the reaction of dimethyl sulphate with silk fibroin in the presence of alkali is entirely different from that occurring in its absence, which, according to Blackburn *et al.* [1941], leads to the introduction mainly of ester-type *O*-methyl which can be entirely liberated by the action of dilute alkali. Rutherford, Patterson & Harris [1940], by exhaustive treatment of fibroin with diazomethane in ether, obtained a product in which (as judged by non-isolative procedures) the tyrosine-phenolic groups were completely etherified, but there was no evidence for methylation of aliphatic —OH groups, most of the extra *O*-methyl being of a type similar to that studied by Blackburn *et al.* [1941].

Since the amino-N content of a hydrolysate of our fully methylated fibroin was little different from that of a hydrolysate of untreated fibroin, it seems unlikely that the reagents employed gave rise to significant *N*-methylation of amino-acid residues in peptide linkage.

Since the positions in the fibroin at which *O*-methyl groups are introduced by methylation appear also to be accessible to acetylation by a procedure [cf. Carr, 1938*b*] that does not seriously degrade the fibroin, it is reasonable to conclude that these (i.e. the —OH groups of nearly all the tyrosine and about one-half of the serine residues) are free in intact fibroin. For the tyrosine, this conclusion is consistent with the phenolic colour reactions of fibroin, and with the data of Rutherford *et al.* [1940] on methylation, of Meyer, Fuld & Klemm [1940] on coupling with diazo compounds, and of Kise & Carr [1936] and Carr [1938*a, b*] on acetylation. Serine residues having free —OH groups might be expected to take part in the alkaline degradation and coupling reactions demonstrated in whole silk by Nicolet & Shinn [1941*a, b*].

The mode of linkage of the serine residues which do not undergo *O*-methylation remains obscure. The present work has not established whether or

not their —OH groups are accessible to acetylation. It is possible that they are not methylated owing to incorporation in 'crystallites' in the fibre, while serine residues in the 'encrusting substance' are reactive [cf. Meyer *et al.*, 1940; Synge, 1943; Huggins, 1943]. Or they might be involved in oxazoline rings of the type postulated by Linderström-Lang & Jacobsen [1941] [cf. Blackburn, Middlebrook & Phillips, 1942]. The present work only demonstrates that the serine residues of silk fibroin fall into two distinct categories with respect to their reactivity with dimethyl sulphate and alkali in the cold. Investigation of the behaviour under these conditions of other proteins and model substances, though technically more difficult owing to their solubilities, may help to elucidate this question.

EXPERIMENTAL

Analytical methods

Estimation of tyrosine. The colorimetric method of Arnou [Block, 1938] was employed.

Estimation of total hydroxyamino-acids. The method of Van Slyke, Hiller & MacFadyen [1941] was modified, by the use throughout of one-tenth of the amounts of reagents which they employed, and by omission of the capryl alcohol. The reagents were added to the outer compartment of the Conway micro-diffusion apparatus. The NH_3 evolved after 1½ hr. gentle rotatory shaking was determined by absorption in 1.0 ml. of 1% boric acid mixture, according to the method of Conway & O'Malley [1942]. Under these conditions we obtained 94–95% recovery of NH_3 (80–120 $\mu\text{g.}$) from ammonium sulphate in 2 ml. half-saturated K_2CO_3 solution. All results have been corrected for this incomplete absorption. Serine under these conditions yielded the theoretical quantity of NH_3 .

Estimation of —OMe. On methylated fibroin and its hydrolysates this was determined by Dr S. Blackburn with the volatile iodide procedure of Baernstein [cf. Blackburn *et al.*, 1941]. Other determinations were made with the standard Pregl procedure by Dr G. Weiler, Oxford, who also carried out carbon and hydrogen determinations.

Estimation of total N. Semimicro-Kjeldahl determinations were employed, using the incineration mixture of Campbell & Hanna [1937].

Estimation of amino-N. The manometric Van Slyke procedure (4 min. reaction time) was employed. Where the sample contained free mineral acid, this was neutralized before analysis with excess of sodium acetate.

Melting points are given uncorrected. Optical rotations were observed in a 2 dm. tube.

Material

Material from the same batch of unweighted silk fabric as was studied by Blackburn *et al.* [1941] was employed throughout this work. The fabric was thoroughly rinsed with cold distilled water before use. Weights were determined after samples had been dried to constant weight over H_2SO_4 at room temp. *in vacuo*.

Methylation of fibroin

A typical experiment is described: 3.284 g. of fabric were cut into small pieces and placed in a bottle with 40 ml. of

N NaOH solution. 0.75 ml. of dimethyl sulphate was added. At 10 min. intervals 20 ml. *N* NaOH solution and 0.75 ml. dimethyl sulphate were added, until altogether 7.5 ml. dimethyl sulphate had been added. The preparation was shaken gently at room temperature the whole time.

At the end, most of the liquor was poured from the fabric, and treatment was repeated. After each treatment a test portion of the fabric was placed in sodium nitrite-acetic acid solution, and the development of yellow colour was observed. This steadily decreased until after four treatments it was practically absent.

When the preparation had to be left between treatments, the fabric was suspended in dilute acetic acid.

Samples removed for determination of —OMe were washed in six changes of distilled water before analysis. The course of methylation is shown in Table 1.

Table 1. *Course of methylation of silk fibroin*

No. of treatments	<i>O</i> -methyl content (CH_3 as percentage of dry wt. of product)
4	1.95
6	2.09
9	2.28
12	2.15

It appears that *O*-methylation is substantially complete after six treatments. The weight of final product (12 treatments), together with that of samples removed during methylation, was 2.96 g. The original material had N, 18.3%, and the final product N, 17.6%. Recovery, based on the N content, was therefore about 85%.

After treatment the fabric was readily creased, had much lower tensile strength, and lacked lustre.

Methylation following acetylation of fibroin

Dry fibroin was acetylated by refluxing in excess of acetic anhydride for ½ hr. The reagent was then washed off with water, and the material subjected to a single methylation treatment as above. The material was then rinsed, dried, and again subjected to acetylation. After four such cycles the product had *O*-methyl (CH_3 as percentage of dry weight) 1.64%, suggesting that the method has no advantages over direct methylation, especially since greater losses resulted.

Acetylation of normal and methylated fibroin

The fabrics were acetylated by refluxing for ½ hr. in acetic anhydride, rinsed off with several changes of distilled water and dried.

The difference between the alkali neutralized by acetylated and unacetylated material on suspending in cold dilute alkali served as a measure of the *O*-acetyl groups introduced.

Approximately 60 mg. of fabric were placed in a stoppered flask in 10 ml. *N*/50 $\text{Ba}(\text{OH})_2$, and kept at room temperature with occasional gentle agitation. Control experiments showed that under these conditions no further alkali was neutralized after 6 hr. After 24 hr. a measured portion of fluid was removed from the flask and titrated with dilute HCl, with the exclusion of CO_2 and with phenolphthalein as indicator. Calculated for the whole volume, the difference from the direct titration of the alkali gave the total base bound by the saponified fibroin together with that neutralized by the acetic acid liberated in the saponification. The results of these experiments are shown in Table 2.

Table 2. *Analyses of silk fibroin after various treatments*

Material	Equivalents expressed in terms of N (1 atom) as a percentage of total N of material analyzed			
	Untreated * material	Same acetylated	Methylated (12 treat- ments)	Same acetylated
Alkali neutralized	5.1	18.4	1.9	6.0
O-methyl content	0.0	0.0	11.4	12.4

Thus for unmethylated fibroin the *O*-acetyl groups introduced are $18.4 - 5.1 = 13.3\%$ (equiv. in terms of N as a percentage of total N of fibroin), while for methylated fibroin the corresponding figure is $6.0 - 1.9 = 4.1\%$. The difference of *O*-acetyl content ($13.3 - 4.1 = 9.2\%$) is in reasonable agreement with the *O*-methyl content of the methylated fibroin, shown in Table 2, where it is expressed in the same units. The decreased binding of alkali by methylated as compared with untreated fibroin is presumably due to methylation of the base-binding phenolic groups of tyrosine residues [Gleysteen & Harris, 1940].

The yields of insoluble material after methylation, acetylation and saponification, expressed on a N basis, are shown in Table 3.

Table 3. *Yields and analyses of fibroin after various treatments*

Treatment	Yield (total N of product as percentage of total N of untreated fibroin)	<i>O</i> -methyl content (—OMe (1 equiv.) expressed in terms of N (1 atom) as percentage of total N of product)
Untreated	100	—
Untreated after saponification	96.7	—
Methylated	86	11.4
Same material after saponification	83	12.6
Acetylated	97	—
Same material after saponification	85.5	—
Methylated and then acetylated	73.6	12.4
Same material after saponification	65.5	11.2

The relative constancy of the *O*-methyl content of the material (Table 3) despite the considerable losses encountered in these treatments suggests that these losses did not involve serious qualitative fractionation of the fibroin, and justifies the assumption that the material lost by solution during processing was not chemically very different from that retained.

Hydrolysis of methylated fibroin

Refluxing fully methylated fibroin for 24 hr. in 6 *N* HCl resulted in considerable de-methylation. Tyrosine colour reactions appeared in the hydrolysate. The —OMe content of the hydrolysate (determined after evaporation to dry-

ness) was 2.3% (—OMe expressed in terms of N (1 atom) as percentage of total N of material), compared with a figure of 11.4% for the material before hydrolysis. The $\text{NH}_3\text{-N}$ liberated by periodate was 10.0% of the total N of the hydrolysate, the corresponding figure for a hydrolysate of untreated fibroin being 10.9%.

Hydrolysis of methylated fibroin for 24 hr. in boiling 6 *N* NaOH reduced the —OMe content (same units—determined after neutralization of hydrolysate with HCl and evaporation to dryness) to 5.1%. Destruction of *O*-methyl-tyrosine would not be expected under these conditions [Rutherford *et al.*, 1940], but when 35 mg. of *O*-methyl-*dl*-serine were refluxed for 24 hr. in excess 6 *N* NaOH with 500 mg. of untreated silk fibroin, only 26%, as measured by an —OMe determination, remained.

Bell [1937] noted removal of ether-methyl during treatment of methylated glycogen with hot hydrochloric acid, and found that *p*-toluenesulphonic acid effected satisfactory hydrolysis without de-methylation. For the present work we have found sulphuric acid to be satisfactory. 70% of the —OMe of 37 mg. *O*-methyl-*dl*-serine was recovered after refluxing with 200 mg. of untreated silk fibroin in excess 6 *N* H_2SO_4 for 24 hr. (SO_4^{2-} was removed from the hydrolysate exactly by precipitation with BaCl_2 . The filtrate and washings were then evaporated to dryness *in vacuo* at room temperature before analysis.)

Analysis of hydrolysates of methylated and untreated fibroin

For comparative purposes, both methylated and untreated fibroins were hydrolyzed by refluxing for 24 hr. in 30 parts by weight of 6 *N* H_2SO_4 . SO_4^{2-} was removed by addition of the calculated quantity of BaCl_2 in aqueous solution, and the filtrate and washings from BaSO_4 were evaporated to dryness *in vacuo* below 40° and made up to known volume in water before analysis. The results are given in Table 4.

Demonstration of O-methyl-tyrosine in hydrolysate of methylated fibroin

O-methyl-tyrosine was obtained as its *N*-acetyl derivative by direct extraction and crystallization after acetylation of the hydrolysate [cf. Synge, 1939].

Methylated fibroin containing 234 mg. N (OMe as N (1 atom) in % of total N, 11.4%) was refluxed for 24 hr. in 25 ml. 6 *N* H_2SO_4 . The resulting hydrolysate was neutralized with NaOH to thymol blue (yellow), concentrated *in vacuo* until crystallization set in, made alkaline by addition of further NaOH and acetylated at 0° by addition of 5 ml. of acetic anhydride and 50 ml. 2 *N* NaOH added in portions with shaking. After keeping alkaline 2–3 hr. the solution was acidified to thymol blue (pink) with 10 *N* H_2SO_4 and extracted five times with 125 ml. portions of B.P. chloroform. The combined chloroform extracts were evaporated to dryness. Crystallization of the residue from water yielded gummy crystals, which were freed from gum by two recrystallizations from chloroform. The final product (61 mg.) had m.p. 147°, not depressed on admixture of authentic *N*-acetyl-*O*-methyl-*L*-tyrosine, $[\alpha]_D^{20} + 51.5^\circ$ (ethanol, $c = 0.9$). Synge [1939] records for *N*-acetyl-*O*-methyl-*L*-tyrosine m.p. 151°, $[\alpha]_D^{20} + 54.3^\circ$, $+ 54.4^\circ$ (ethanol, $c = 1.3$). (Found: C, 61.0; H, 6.53; N, 4.8; OMe, 12.4%; acid equiv. wt. 250. Calc. for $\text{C}_{12}\text{H}_{15}\text{O}_4\text{N}$: C, 60.8; H, 6.33; N, 5.9; OMe, 13.1%; acid equiv. wt. 237.)

Table 4. *Analytical data on hydrolysates of methylated and untreated fibroins*

	Percentage of total N of material analyzed			
	Methylated fibroin	Untreated fibroin		Reference
		Present work	Other authors	
		Before hydrolysis		
—OMe (1 equiv. = 1 atom N)	10.3	—	—	
		After hydrolysis		
(1) —OMe (1 equiv. = 1 atom N)	9.15	—	—	
(2) Amino-N	90	92	—	
(3) Tyrosine N	0.35	4.5	4.6*	Meyer, Fuld & Klemm [1940]
(4) NH ₃ -N + NH ₃ -N liberated by periodate	7.0	11.9	—	
(5) NH ₃ -N	1.45	1.0	—	
(6) Hydroxyamino-acid N (by difference of (4) and (5))	5.55	10.9	10.8*	Nicolet & Saidel [1941]

* N content of the fibroin analyzed by these workers assumed to be 18.3%.

Demonstration of O-methyl-serine in hydrolysate of methylated fibroin

O-methyl-serine was separated as its *N*-acetyl derivative by partition chromatography on silica gel [Gordon *et al.*, 1943*a*]. Control experiments with authentic *N*-acetyl-O-methyl-*dl*-serine [Synge, 1939] showed that on partition chromatograms employing B.P. chloroform, ethyl acetate or 30% propanol-cyclohexane as developing solvent, it is not separable from acetylalanine. With 17% butanol-chloroform, *N*-acetyl-O-methyl-serine travels as a band sufficiently faster than acetylalanine to permit the recognition of its presence, though not fast enough to permit satisfactory separations. However, with 1% butanol-chloroform, it moves as a separate band having *R* [Martin & Synge, 1941] approximately 1.5 times that of acetylalanine.

On the basis of this information the following experiments were carried out: Approximately 10 mg. samples of fully methylated (as used in previous section) and untreated fibroin were hydrolyzed with sulphuric acid, and the hydrolysates evaporated to dryness *in vacuo* below 40° after removal of SO_4^{2-} as described above. The hydrolysates were then analyzed according to the procedure given by Gordon *et al.* [1943*a*]. Pelargonin chloride was employed as indicator. During the development with 17% butanol-chloroform it was noted that with the material from methylated, as compared with that from untreated fibroin, the bands corresponding to *N*-acetyl-tyrosine and *N*-acetyl-serine [Gordon, Martin & Synge, 1943*b*] were greatly reduced in intensity, while the band corresponding to acetylalanine was broader and exhibited a 'faint front', as with the synthetic mixtures of acetylalanine and *N*-acetyl-O-methyl-serine studied above. The bands corresponding to acetyl-glycine did not differ in appearance.

The values found for 'alanine' (N as percentage of total N of fibroin) were 24.5% for untreated and 30.8% for methylated fibroin. The difference (6.3%) is consistent with the O-methyl-serine value suggested by the data given above. Bergmann & Niemann [1937] recorded for alanine in silk fibroin a corresponding figure of 21.8%. They assumed their fibroin to contain 19.0% N.

After titration the 'alanine' fractions were acidified and examined on 1% butanol-chloroform chromatograms. That

from methylated fibroin showed two bands, similar to those given by the synthetic mixtures described above, while that from untreated fibroin gave a single band (acetylalanine).

An attempt was then made to isolate O-methyl-serine as its acetyl derivative. Since the only authentic material available for comparison [Synge, 1939] was racemic, the acetylation was conducted under racemizing conditions [du Vigneaud & Meyer, 1932]. Complete racemization was not, however, achieved, and the crystallization of the final product may have been interfered with by partial racemization thus induced.

275 mg. of the same methylated fibroin were hydrolyzed with sulphuric acid, freed from SO_4^{2-} with BaCl_2 and evaporated to dryness as above. The product was treated with 5 ml. 2.4*N* NaOH and 2 ml. acetic anhydride, the mixture being kept at 40° for 15 hr. The mixture was then cooled, made alkaline to thymolphthalein by addition of 12 ml. 3*N* NaOH, and kept at room temperature for 2 hr. to saponify O-acetyl groups. The mixture was then acidified with 10*N* H_2SO_4 and extracted with five 100 ml. portions of 17% butanol-chloroform. The material in the combined extracts was subjected to chromatographic analysis with 17% butanol-chloroform as developing solvent. The resulting 'alanine' band was subjected to further analysis, by development with 1% butanol-chloroform. Of the two bands so obtained, the faster yielded 20 mg. of material. Treated with ethyl acetate-light petroleum this yielded 11 mg. of partly crystalline gummy material having m.p. 128–130°, $[\alpha]_D^{25} - 12^\circ$ (ethanol, *c* = 0.4). The material depressed the m.p. of acetyl-*L*-alanine (m.p. 126°) and of acetyl-*dl*-alanine (m.p. 133°) on admixture by more than 30°. It proved difficult to recrystallize. OMe was determined on the material recovered from the polarimetric determination after removal of ethanol by evaporation *in vacuo* over H_2SO_4 . (Found: OMe, 7.3%. Calc. for $\text{C}_6\text{H}_{11}\text{O}_4\text{N}$: OMe, 19.3%.)

SUMMARY

1. Repeated treatment of silk fibroin with dimethyl sulphate in *N* NaOH at room temperature results in O-methylation of nearly all its tyrosine residues and about one-half of its serine residues.

2. The groups accessible to étherification in this

way are also accessible to acetylation when the fibroin is treated with boiling acetic anhydride.

3. The existence is thus established of two categories of serine residue in silk fibroin, differing in their accessibility to etherification by dimethyl sulphate.

We are grateful to Dr Herbert E. Carter for a gift of *O*-methyl-*dl*-serine.

We wish to thank the Director and Council of the Wool Industries Research Association and the International Wool Secretariat for permission to publish this work, and Dr S. Blackburn for carrying out —OMe determinations.

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The Relative Efficacy of Calcium Carbonate and Calcium Phosphate in Preventing Rickets in Rats

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(Received 2 August 1943)

Present knowledge of the metabolism of calcium and phosphorus suggests that calcium phosphate is likely to be more effective as a calcifying agent than the carbonate. Since the question of dietary supplies of calcium became topical with the proposal to add carbonate to bread, some experiments were begun to compare the power of the carbonate and the phosphate to prevent rickets in rats. The applicability of the findings to human rickets will be discussed after the presentation of the results.

EXPERIMENTAL

General plan. The general plan of the experiments was to place young rats on diets comprising mostly flour to which were added varying amounts of CaCO_3 or CaHPO_4 ; after 5 weeks on these diets the ash content of both femurs of each animal was determined. The rats at the beginning of the experiment were about 6 weeks old and weighed about

50 g.; they were kept in separate cages throughout the experiment and weighed weekly. Each group receiving a given level of Ca contained five animals. CaCO_3 was given as the preparation used to fortify bread, i.e. *creta preparata* B.P. The highest amount of each salt added to the diet was equivalent to 3% of Ca, i.e. 7.5% CaCO_3 or 10.1% CaHPO_4 .

Diets. In most of the experiments the basal diet consisted of flour to which was added 1% NaCl. The flour was either white (70% extraction) or National Wheatmeal (85% extraction). When white flour was used the rats were given supplements of aneurin and riboflavin equivalent to 10 μg . daily of the former and 4 μg . of the latter. No supplement was given with the wheatmeal flour. For comparative purposes one experiment was carried out with a maize diet similar to that commonly used for producing rickets in rats. This basal diet contained maize meal 76 parts, gluten 20 parts.

To the basal flour or maize diets were added 1% NaCl and varying amounts of the calcium salts. No further additions were made. With three parts CaCO_3 , the maize meal