ments. The possibility exists that they may be converted into substances which have actions on the fatty liver different from that of the amino-acids in the original form. An example of this reversal of action is given by the work of Singal & Eckstein [1941], who found that whereas cystine accelerated liver-fat deposition, cystine betaine had the opposite action, and another substance having a lipotropic action was dithiodiglycollic acid.

It is to be regretted that conditions did not allow of our continuing this investigation, and consequently our results are presented in an incomplete form.

SUMMARY

1. Fourteen pure amino-acids were investigated with a view to ascertaining whether or not they had any effect on the deposition of fat in the livers of rats on diets high in fat and low in lipotropic factors.

2. Tryptophan was found to possess a small lipotropic action in one experiment, and tyrosine to

possess a small but questionable action on the fat- and cholesterol-fatty livers.

3. Glutamic acid was found to exert a lipotropic action on cholesterol- but not on fat-fatty livers.

4. The lipotropic actions of various protein fractions were studied. Evidence was obtained that some factor which affects liver-fat deposition, other than cystine, methionine and tyrosine, is present in the protein molecule. This factor can be concentrated in the butanol-soluble amino-acid fraction. Fractionation by the copper-salt method effected further concentration of the unknown factor and indicated the possible presence of a substance which accelerates liver-fat deposition. Conflicting results have been obtained in experiments with gelatin, and in one experiment with caseinogen fractions, and more work is necessary to elucidate the problem completely.

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A Biological Method for Estimation of Pantothenic Acid with Rats, in which Wheat Germ is included in the Basal Diet

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Investigations on the B₂ group of vitamins made with chicks fed on a heated diet of mixed grains, and with rats on synthetic diets supplemented by purified aneurin (vitamin B₁), riboflavin, and pyridoxine (vitamin B₆), have led to the discovery that the filtrates obtained when extracts of yeast, liver or rice bran are treated with Fuller's earth contain substances essential for growth of these animals

[Lepkovsky, Jukes & Krause, 1936; Elvehjem & Koehn, 1935]. These substances, generally known as filtrate factors, were called factor 2 by Lepkovsky and his colleagues. Chicks deprived of filtrate factors are checked in growth and develop a characteristic dermatitis; young rats also show subnormal increase in weight and a less specific skin affection with greying of the pigmented fur. In due course

it was discovered that the filtrate factor fraction contained a substance identical with 'pantothenic acid', a compound found necessary for the growth of certain strains of yeast [Williams, Lyman, Goodyear, Truesdail & Holaday; 1933]. For a concise account of the gradual discovery to date of the different components of the vitamin B₂ complex reference should be made to the review by Lepkovsky [1942].

Before the identification and synthesis of pantothenic acid was accomplished [Williams & Major, 1940], Jukes [1937] had measured the 'filtrate factor' potency of different foods by feeding them to chicks maintained on the heated grain diet. The criterion adopted was rate of weight increase, a preliminary attempt to use degree of cure of dermatitis having proved unsatisfactory.

There is no record of any attempts to use rats for the biological estimation of pantothenic acid, although the growth-promoting activity of certain materials fed to rats as supplements to the so-called 'filtrate-deficient' diet has been measured in this laboratory [Copping, 1943]. The results cannot be applied directly to the pantothenic acid content of these materials, since it is now certain that 'filtrate factor deficiency' is not a deficiency of pantothenic acid alone, but also of at least one other growth-promoting substance [see Lepkovsky, 1942].

In an attempt to estimate a vitamin by biological method, in which the growth of a young animal is adopted as criterion, the first requisite is a basal diet which shall contain, in optimum amounts, all other essential nutrients. We therefore looked for an addition to the 'filtrate-deficient' synthetic diet which should supply the additional unknown growth 'filtrate factors' without pantothenic acid. Our first choice was a whole liver extract in which we hoped to destroy the pantothenic acid present by acid or alkaline hydrolysis. This proved unsuccessful (see below, p. 494), and we finally chose wheat germ. Bakke, Aschehoug & Zbinden [1930] noted the development of greyness of rats with pigmented fur on a diet in which wheat germ supplied the B vitamins, and ascribed the effect to toxicity of the wheat germ. Miss A. M. Copping (private communication) made a similar observation while measuring the growth response of young pied rats to doses of wheat germ added to a 'filtrate-factor deficient diet', the rats becoming grey while still growing. It was assumed that this was due to the absence of pantothenic acid in wheat germ, a material which nevertheless contained considerable amounts of the other additional growth factor or factors. Preliminary tests based on this assumption have met with some success, and in this paper is given an account of these and of the development of a biological method suitable for estimation of pantothenic acid, in which the growth of young rats is the criterion adopted.

EXPERIMENTAL

The rats, all males of the usual Lister pied strain, were weaned on to a basal diet deficient in all B₂ vitamins for 1-2 weeks before receiving the test doses. The basal diet was that employed at the Lister Institute, and, except for a few preliminary tests in which starch was given, the carbohydrate was present in the form of sucrose. The composition was as follows: sucrose 60, casein 20, arachis oil 12, salts 5 and lard 3 parts. Vitamin B₁ as pure aneurin hydrochloride was given daily, 15 µg. to each rat, and cod-liver oil to supply vitamins A and D. The diet was unsupplemented with any B₂ vitamins for a period of 2 weeks in the hope that any variations due to reserve of stores of these vitamins in the young rat's body might be lessened by this procedure.

The rats, which usually weighed 40-55 g. at the beginning of the tests, were subsequently weighed three times a week. At the beginning of the test period they were put into separate cages of the usual pattern, distributed evenly by weight and litter among the various groups. They then received supplements of B₂ vitamins other than pantothenic acid in pure form. Two solutions of mixed vitamins were prepared. The first contained 30 µg. aneurin, 30 µg. pure vitamin B₆ (pyridoxine) and 50 µg. riboflavin in the daily dose of 2 ml. The second was stored in a dropping bottle which delivered, in the daily dose of 2 drops, 1 mg. inositol, 1 mg. nicotinic acid and 3 mg. choline chloride. Although it is doubtful whether the rat requires inositol and nicotinic acid, their inclusion in the supplements seemed desirable.

The basal sugar diet was put into heavy glass dishes and pressed down firmly to avoid scattering. If scattering took place, water was added, and any diet remaining unconsumed after 24 hr. was discarded. Fresh dry diet was given daily in weighed amount to supply a slight excess over the rat's requirements.

When wheat germ was given, the daily dose was weighed into a heavy glass dish, moistened with the drops of inositol, nicotinic acid and choline chloride; this mixture was usually consumed readily before any basal diet was given. With the larger doses of wheat germ, extra water was needed. The doses of vitamin B₁, vitamin B₆ and riboflavin were given in the same dish. The doses of pure pantothenic acid, given to the control series of rats, were from a solution containing 100 µg./ml. of calcium-*D*-pantothenate.

The rats were kept on the test doses for 4 weeks, weighed three times a week, and any symptoms noted as they appeared. The absence of symptoms was also recorded when the test was finished.

RESULTS

Experiments with whole-liver extract

Since pantothenic acid is reported to be unstable to acid and alkaline hydrolysis it was expected that a source of the unidentified factors free from it might be obtained by subjecting liver extract to these treatments.

Accordingly a whole-liver extract was adjusted to pH 0 by the addition of conc. H₂SO₄ and boiled on a sand bath for 10 hr. The resulting dark solution was neutralized with baryta, filtered under pressure,

Table 1. *Growth of rats receiving supplements of liver extract with or without added pantothenate*

Exp.	Supplementary dose, daily	No. of rats	Average weekly weight increase (g.)			
			Week 1	Week 2	Week 3	For whole period
1	Whole liver extract $\equiv$ 6 g. fresh liver	6	27.0	25.7	24.0	25.6
	„ acid treated	8	20.0	17.8	18.9	18.9
	„ acid treated + 75 μ g. pantothenate	7	22.0	21.6	21.2	21.6
2	Whole liver extract $\equiv$ 6 g. fresh liver	3	33.0	24.0	27.9	28.3
	„ alkali treated	4	30.7	22.5	21.2	24.8
	„ alkali treated + 75 μ g. pantothenate	4	33.0	28.8	28.2	30.0

extracted twice with ether in an attempt to remove bitter-tasting products of hydrolysis and freed from ether by evacuation. The growth-promoting effect of this extract with and without pantothenic acid was compared with that of the untreated extract, but it was clear that the pantothenic acid had been only partially destroyed by the treatment, since addition of pantothenate to the treated extract gave but a small improvement in weight increase which was not statistically significant (Table 1, Exp. 1).

Alkaline hydrolysis was carried out in solution raised to pH 11.5 by addition of baryta. The first attempts produced a dark-coloured product with bitter taste, which was badly taken by the rats and caused diarrhoea. Finally, a palatable and innocuous product was obtained after hydrolysis for 5 hr. at this pH. The growth on the dose given was, however, sufficiently near to that on the original liver extract to indicate that little destruction of

pantothenic acid had occurred, and the increased growth produced by the addition of pantothenic acid was too small to form the basis of an assay, although statistically significant (see Exp. 2, Table 1).

Experiments with wheat germ

To find out whether wheat germ was deficient in pantothenic acid but contained the additional growth factors, 23 rats from six litters were divided into three groups and given daily doses, respectively, of 1 g. wheat germ, 1 g. wheat germ plus 150 μ g. calcium-*d*-pantothenate, and 150 μ g. calcium-*d*-pantothenate only.

The results summarized in Table 2 show quite clearly that when 1 g. wheat germ daily was fed with 150 μ g. calcium-*d*-pantothenate, an amount known [cf. Unna, 1940] to be in excess of the rat's requirements, the average weekly weight increase (27.1 g.) was greater than with pantothenic acid

Table 2. *Growth of rats receiving supplements of wheat germ with or without added pantothenate*

Exp.	Supplementary dose, daily	No. of rats	Average weekly weight increase (g.)			
			Week 1	Week 2	Week 3	For whole period
3	1 g. wheat germ	7	22.6	19.4	15.6	17.3
	1 g. wheat germ + 150 μ g. Ca- <i>d</i> -pantothenate	8	24.4	26.0	27.6	27.1
	150 μ g. Ca- <i>d</i> -pantothenate	8	22.1	17.9	17.3	19.1
4	0.5 g. wheat germ	7	18.4	14.4	9.8	13.6
	0.5 g. wheat germ + 100 μ g. pantothenate	8	27.5	27.4	29.2	30.3
	1 g. wheat germ	8	22.1	13.3	12.9	15.7
	1 g. wheat germ + 100 μ g. pantothenate	8	27.4	24.0	26.6	28.3
	2 g. wheat germ	8	23.8	18.6	20.0	21.6
	2 g. wheat germ + 100 μ g. pantothenate	8	26.7	25.9	23.9	25.7
	Whole liver extract $\equiv$ 6 g. fresh liver	5	31.6	23.8	30.1	28.5

Table 3. *Growth of rats receiving supplements of pantothenate, liver extract and/or wheat germ*

Exp.	Supplementary dose, daily	No. of rats	Average weekly weight increase (g.)			
			Week 1	Week 2	Week 3	For whole period
5 (a)	Liver extract $\equiv$ 12 g. fresh liver	4	34.0	30.8	39.6	34.7
	Liver extract $\equiv$ 6 g. fresh liver + 1 g. wheat germ	4	32.5	34.1	36.0	35.9
	Liver extract $\equiv$ 6 g. fresh liver + 2 g. wheat germ	3	32.7	30.3	36.0	34.4
5 (b)	Liver extract $\equiv$ 6 g. liver	8	29.1	28.1	29.5	28.3
	Liver extract $\equiv$ 3 g. liver + 0.25 g. wheat germ	8	30.9	25.1	28.3	29.1
	Liver extract $\equiv$ 3 g. liver + 0.5 g. wheat germ	8	28.4	27.8	28.4	28.5

All rats received 100 μ g. Ca-*d*-pantothenate daily.

alone (19.1 g.), thus affording evidence for the presence in wheat germ of the additional unknown growth-promoting substances. Without pantothenic acid the growth-promoting effect was considerably reduced (average weekly weight increase 17.3 g.), demonstrating the deficiency of wheat germ in this substance (Exp. 3, Table 2).

In order to determine how much wheat germ was needed to meet the rat's daily requirements, doses of 0.5, 1 and 2 g. daily were chosen and tested with and without 100 μ g. pantothenic acid. Fifty-two rats from ten litters were used in this test, and a group of rats receiving whole-liver extract served as a positive control (Exp. 4, Table 2). These results showed that 0.5 g. wheat germ was as effective as 1 or 2 g. in growth-promoting power when supplemented with 100 μ g. pantothenic acid, the average weekly weight increases being 30.3, 28.3 and 25.7 g. respectively. Increasing amounts of wheat germ fed without pantothenic acid gave increasing growth rates. This might be explained by the assumptions: (a) that 0.5 g. wheat germ contains more than the rat's daily requirement of the additional factors, and/or (b) that wheat germ also contains a small amount of pantothenic acid. The maximum weekly weight increase in this series, 30.3 g. when 0.5 g. wheat germ was supplemented with 100 μ g. pantothenic acid, is comparable with that obtained, 28.5 g., when whole-liver extract equivalent to 6 g. of fresh liver was given.

In order to determine whether wheat germ supplies all the additional growth factors other than pantothenic acid, comparison was made between wheat germ and whole-liver extract, which is the richest known source of these factors and was used by Macrae, Todd, Lythgoe, Work, Hind & El Sadr [1939]. For this test 34 male rats from 11 litters

were used. If wheat germ and whole-liver extract each contain all these additional factors and were given in adequate amounts, either should give maximum growth, and any mixture of the two should be equivalent to a corresponding amount of either. This was tested when 2 g. wheat germ were compared with whole-liver extract equivalent to 6 g. fresh liver, and also when 0.5 g. wheat germ was compared with liver extract equivalent to 3 g. fresh liver, calcium-*d*-pantothenate and other vitamins being fed at optimum doses to all animals. The last tests were important, because, if differences existed between wheat germ and liver extract with regard to the relative proportions of the hypothetical additional factors, such differences would be more apparent at lower dosage levels. No evidence, however, was found for any qualitative differences or, within the limits of the test, for differences in the proportions of the additional factors. The results are given in Table 3.

Effect of additions of different amounts of pantothenic acid to 0.5 g. wheat germ. A daily dose of 0.5 g. of wheat germ was chosen as suitable, and determinations were made of the effect on growth rate of additions of 0, 12.5, 25, 50, 100 and 200 μ g. daily of calcium-*d*-pantothenate. These results are divided into three sections, since litters containing six male rats were not always obtainable for all the levels to be tested at once.

The pantothenate was made up in two solutions containing, in 1 ml., 25 and 50 μ g. calcium-*d*-pantothenate, respectively, and a little chloroform was added. The requisite amounts were delivered into the dose dish before it was placed in the cage.

The results in Table 4 show that 12.5–50 μ g. pantothenate daily were suboptimal doses, and that the optimal dose was probably between 50 and

Table 4. *Growth of rats receiving supplements of wheat germ with varying amounts of added pantothenate*

Exp.	Supplementary dose, daily	No. of rats*	Average weekly weight increase (g.)				
			Week 1	Week 2	Week 3	Week 4	For whole period
6 (a)	0.5 g. wheat germ	18	16.1	11.5	9.3	9.5	11.6
and	0.5 g. wheat germ + 12.5 μ g. Ca- <i>d</i> -pantothenate	18	19.8	14.4	12.9	14.1	15.3
6 (c)	0.5 g. wheat germ + 25 μ g. Ca- <i>d</i> -pantothenate	19	20.2	20.2	18.7	10.9	17.5
	0.5 g. wheat germ + 100 μ g. Ca- <i>d</i> -pantothenate	18	27.8	26.6	27.2	29.6	27.8
	Liver extract $\equiv$ 6 g. fresh liver	6	26.5	17.9	18.7	24.5	26.9
6 (b)	0.5 g. wheat germ	14	16.2	10.4	9.1	9.1	11.2
and	0.5 g. wheat germ + 25 μ g. Ca- <i>d</i> -pantothenate	13	24.0	18.1	15.9	17.9	19.1
6 (c)	0.5 g. wheat germ + 50 μ g. Ca- <i>d</i> -pantothenate	14	23.9	23.3	23.6	23.2	23.5
	0.5 g. wheat germ + 100 μ g. Ca- <i>d</i> -pantothenate	14	29.2	24.2	27.3	29.3	27.5
6 (c)	0.5 g. wheat germ	8	15.6	11.8	9.5	8.3	11.3
	0.5 g. wheat germ + 12.5 μ g. Ca- <i>d</i> -pantothenate	8	20.0	16.8	13.6	13.2	15.9
	0.5 g. wheat germ + 25 μ g. Ca- <i>d</i> -pantothenate	8	22.9	19.7	16.2	15.2	18.5
	0.5 g. wheat germ + 50 μ g. Ca- <i>d</i> -pantothenate	10	22.7	26.3	23.7	23.3	23.0
	0.5 g. wheat germ + 100 μ g. Ca- <i>d</i> -pantothenate	8	31.5	25.5	29.1	28.3	28.6
	0.5 g. wheat germ + 200 μ g. Ca- <i>d</i> -pantothenate	4	26.3	25.5	22.3	27.1	25.3

* The results given in Exp. 6 (c) for doses of 12.5, 25, 50 and 100 μ g. Ca-*d*-pantothenate are included in the averages given for these doses in Exps. 6 (a) and 6 (b).

100 μg . This figure is in accord with the figure of 80 μg . daily, given by Unna [1940] as the optimal daily dose for rats. The same result emerges from Fig. 1, in which a fairly smooth curve was obtained when the average weekly weight increases calculated from the results of Exp. 6 (c), etc. in Table 4 were plotted against doses of pantothenate. If the

Fig. 1. Curve showing relation of average weekly rate of weight increase in young rats to the daily dose of Ca-d-pantothenate added to a basal diet containing other B vitamins, 0.5 g. wheat germ being given daily to each rat.

logarithms of the doses are plotted, the curve approximates to a straight line up to doses of about 100 μg . Where possible, rats from the same litter were used in the comparison of these doses, and in one series controls were included receiving a 6 g. equivalent of whole-liver extract (Exp. 6 (a)). Since litters containing six or more male rats occurred infrequently, comparison of the effects of the addition of 0, 12.5, 25 and 100 μg . in Exp. 6 (a), and of 0, 25, 50 and 100 μg . pantothenate in Exp. 6 (b) were made with litters containing four male rats only.

Occurrence of symptoms of deficiency of pantothenic acid

The symptoms noted when insufficient doses of pantothenic acid were given were greying of the naturally pigmented portions of the fur and also a generalized soiling and staining of the fur with an orange yellow substance, mainly in the hind ventral regions. The former is considered characteristic of pantothenic acid deficiency, the latter, which is due to a porphyrin body, has been noted in deficiency of riboflavin as well as in that of the filtrate factor of the vitamin B₂ complex [Chick, Macrae & Worden, 1940]. The above symptoms, which appeared to be aggravated when a small dose of

pantothenate (12.5 μg . daily) was given, are possibly due to a greater strain caused by the increased growth stimulated; they tended to disappear when the rats received 100 μg . daily of Ca-d-pantothenate.

In addition, there was a tendency for the normal jet black patches of fur of all rats to turn a rusty colour after they had received wheat germ, a change not observed when whole-liver extract was given.

DISCUSSION

The use of rats for the estimation of pantothenic acid in natural materials has advantages over the microbiological method, since for the latter the complete extraction of the pantothenic acid is needed in a form in which it can be utilized by bacteria. The presence of 'bound' forms of pantothenic acid has been suggested by the work of Pennington, Snell & Williams [1940], of Waisman, Henderson, McIntire & Elvehjem [1942] and of Strong, Feeney & Earle [1941]. If it may be assumed that the rat can absorb pantothenic acid from all those forms of combination from which it is available to other animals, estimation by the method here described is particularly suitable for avoiding this difficulty.

The successful development of the method on the assumption that wheat germ supplies ample amounts of the 'additional factors' is to be taken as further proof of their existence, and the magnitude of the increase in growth rate ascribable to them suggests that they play a major part in the nutrition of the rat.

SUMMARY

1. Wheat germ has been found to contain adequate amounts of the one or more unidentified factors required by the rat, and is comparatively deficient in pantothenic acid.

2. These facts have been made the basis of a method for the estimation of pantothenic acid by the growth of young rats.

3. Addition of pantothenic acid to a basal synthetic diet supplemented by vitamin B₁ (aneurin), vitamin B₆ (pyridoxine), riboflavin, inositol, choline, nicotinic acid, cod-liver oil and wheat germ was followed by an increase in the growth rate proportional to the logarithm of the amount fed.

4. The optimum daily dose of calcium-d-pantothenate for a young rat (40–150 g. weight) appears to lie between 50 and 100 μg .

In conclusion we acknowledge the personal grant to one of us (J. S. D. B.) from the Medical Research Council, and gifts to the Division of Nutrition of calcium-d-pantothenate from Messrs G. Merck, Rahway, N.J., of pure vitamin B₁ and riboflavin from Roche Products Ltd. and of liver extract from Glaxo Laboratories Ltd. We also wish to thank Dr H. Chick for helpful advice and Miss Ruby Leader for assistance in the care of the experimental animals.

Appendix: Statistical Analysis

By J. O. IRWIN AND G. N. JENKINS

By analysis of variance, the differences from litter to litter of the animals in Exp. 6 (c) were tested and found not to be significant. Hence it is justifiable to estimate the standard errors of mean responses or of slope from the variance in growth of animals (not littermates) on the same dose.

	<i>b</i>	<i>ob</i>	Ratio
Average increase in response (i.e. body-weight increase/week) when dose is doubled	4.26	1.12	3.80
Average increase in response when dose is increased tenfold	14.16	3.70	3.80

The standard error is based on 28 degrees of freedom and the slopes are therefore highly significant. The curve relating the logarithm of the dose to the response shown by rate of weight increase shows no significant departure from linearity.

Ignoring the addition to the error of any estimation due to the error of the estimate of slope, which may, of course, be considerable, the limits of error for one estimation with

ten rats on the standard and ten on the test substance would be:

$p=0.99$	64 to 157%
$p=0.95$	55 to 181%

This basis is comparable with that used in the British Pharmacopoeia Commission Report on the Accuracy of Biological Assays [1936]* and the accuracy found with that usually obtained in biological assays.

For Exps. 6 (a) and 6 (b) the results are as follows:

Exp.	Slope <i>b</i>	<i>ob</i>	Ratio	Limits of error ($p=0.99$) for 10 rats on each dose
6 (a)	4.30	0.76	5.7	61 to 164%
6 (b)	4.19	1.02	4.1	64 to 157%

The errors in the result are increased if the period of dosing is reduced from 3 to 4 weeks, although the results are still significant.

* *Gen. Med. Coun. Brit. Pharm. Com. Report*, p. 10 [1936].

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Intermediate Stages in the Metabolic Conversion of Benzpyrene to 8-Hydroxy-benzpyrene in Mice

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Chalmers & Crowfoot [1941] showed that after administration of benzpyrene to rats and mice a small amount of monohydroxybenzpyrene could be isolated from the faeces. Berenblum, Crowfoot, Holiday & Schoental [1943] identified this as 8-hydroxy-3:4-benzpyrene, and established the presence of 3:4-benzpyrene-5:8-quinone in the faeces. Intermediate metabolites, 'BPX' and 'BPF' could

be demonstrated by a blue fluorescence of the bile [Peacock, 1936, 1940] and a blue-green fluorescence of the faeces [Chalmers, 1938] respectively, both distinguishable from the violet fluorescence of the parent hydrocarbon in organic solvents. Doniach, Mottram & Weigert [1943 *a, b*] found that the blue 'BPX' fluorescence changed to the blue-green 'BPF' fluorescence after the contents of the small