ESTIMATION OF STERILIZING VALUES OF PROCESSES AS APPLIED TO CANNED FOODS. II.*—Packs Heating by Conduction: Complex Processing Conditions and Value of Coming-up Time of Retort

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A method is presented for estimating the F_C value, for conduction packs, of a heating process of any degree of complexity. As a corollary, the value of the coming-up time of the retort may be estimated in terms of time at retort temperature.

Heat flow in some canned foods is entirely by conduction and the can behaves during both heating and cooling as a homogeneous solid obeying the known laws of heat conduction.[1] It is thus possible to calculate the temperature at any point in the can at any time during a process, given the initial temperature, the surface temperature, the dimensions of the can, the heat conductivity and the specific heat (combined as the diffusivity) of the contents. The surface temperature (the processing temperature and the cooling temperature) may be varied during the process in any way whatever, and the only result of complex processing conditions is an increase in the labour of computation.

The course of temperature at any point in the can—in particular the centre, the point of greatest temperature lag—may be followed during heating and cooling by direct observation, usually by means of a thermocouple, but if this method alone is relied upon each change of conditions demands a new set of observations. The convenience of being able to determine the 'heat-penetration curve' by calculation is therefore apparent.

When the 'plot' of the central temperature is available either by observation or calculation, the value of the process for the centre of the can, the F_C value,[2] may be determined graphically by the method of Bigelow *et al.*[3] or arithmetically by the method of Patashnik.[4] In both these methods the central temperatures are converted to lethal rates and these are integrated over the whole time of processing and cooling—in the first method by measurement of area and in the second by Simpson's rule for approximate calculation of areas—to give the F_C value of the process. The lethal rate at any temperature is the sterilizing value of unit time at that temperature in terms of time at 250° F, and the F_C value is the time at 250° F that would have the same sterilizing value as the whole course of temperature at the centre of the can.

When processing conditions are simple, i.e. the processing temperature is constant and the change to cooling is rapid, the F_C value of a process may be calculated directly (given the necessary data) and with little labour by the method of Ball[5] or Gillespy.[2]

It is considered worth while to place on record a method, together with the necessary Tables, whereby the course of temperature at the centre of a can of food heating by conduction may be calculated for a process of any degree of complexity. The method is adapted from that of Riedel,[6] with new Tables; the derivation is given in the Appendix. There is a limitation that all changes in external temperature shall be either instantaneous or rectilinear in relation to time.

It is assumed that the rate of penetration of heat into the can is known in the form of the factor f, which includes the dimensions of the can and the heat diffusivity of the contents.[2] The factor f may be determined experimentally for the food in question under simple processing conditions for one size of can. From this the value of f for any other size of can may be calculated.[2] The factor f, rather than the diffusivity, was chosen as the basic factor in the calculation because, being used in other methods of process calculation, it is often already known. Besides f, it is necessary to know h/d, the ratio of the height of the can to the diameter, excluding the seams.[2] When the course of temperature at the centre of the can has been determined, the conversion to lethal rates depends on the value of z considered appropriate for the food in question.[2] The factor z defines the slope of the thermal death-time curve, assuming a first-order relationship between temperatures of heating and the logarithms of times of heating that give equivalent degrees of destruction of bacterial spores.

For the integration of the lethal rates, the method of Patashnik[4] is adopted, and it is essential that the central temperature be estimated at equal time intervals. Simpson's first (trapezoidal) rule is used, and, as the first and last ordinates are very small, simple addition

* Part I: *J. Sci. Fd Agric.*, 1951, **2**, 107

only is required. The accuracy of this approximate integration depends on the smallness of the constant time interval. It is suggested that intervals of $f/10$ minutes are sufficiently small in most cases. Simpson's other rules (second- and third-degree parabolae) are available, but the greater accuracy is hardly justified in view of the usual uncertainty in the value of z.

Data required

Heat penetration.—The value of f for the can in question, and the relative dimensions h/d.

Processing history.—(i) The initial temperature, T_0, of the contents of the can, assumed to be uniform; (ii) the history of retort temperature, i.e. the time and magnitude of each instantaneous jump or fall (negative jump), including the time at which cooling starts and the temperature of the cooling water, and the time of start and end, and the magnitude, of each linear rise or fall. It is assumed that the initial external temperature is T_0 [from time $(-\infty)$ to time (0), the start of the process].

Procedure

Tabulate processing history under the headings:

θ Time, in minutes, from zero time, at which each change in the course of retort temperature occurs (jump or break)

t θ/f

T_R Retort temperature at time θ (all temperatures are in ° F)

J Magnitude of each jump in temperature

p Rate of change of T_R between each pair of 'breaks'. If consecutive breaks are specified at θ_i (or t_i) and θ_{i+1} (or t_{i+1}), the corresponding values of T_R at these times being T_R and $T_{R_{i+1}}$, then p_i is $f(T_{R_{i+1}} - T_{R_i})/(\theta_{i+1} - \theta)$ or $(T_{R_{i+1}} - T_{R_i})/(t_{i+1} - t_i)$

B Value of each break. B_i at time θ_i (or t_i) is given by $p_i - p_{i-1}$

Calculation of central temperatures.—It is required to find the central temperature at intervals of $f/10$ minutes, i.e. at times $t = 0, 0{\cdot}1, 0{\cdot}2 \ldots$ Some of the earlier times may be omitted as the central temperatures will have negligible lethal rates. The calculation is stopped when the centre has again fallen to a temperature with a negligible lethal rate.

Make a Table with columns for t, T_R, each J and each B in chronological order, T and L. Start t at 0·5, say (according to conditions). Enter the obvious values of T_R, whenever $p = 0$, and calculate the others as follows:

If, from the Table of processing history, T_R at times t_i and t_{i+1} has the values T_{R_i} and $T_{R_{i+1}}$ respectively, and p for this interval is p_i, then T_R at time t $(t_i < t < t_{i+1})$ is $T_R + (t - t_i)p_i$. Fill in each J column as follows: If J_i occurs at t_i, then at each time $t(> t_i)$ enter $J_i \times \Phi(t - t_i)$, from Table III for the appropriate value of h/d. Head the column with the opposite sign from that of J_i in Table of processing history.

Fill in each B column as follows: If B_j occurs at time t_j, then at each time $t(> t_j)$ enter $B_j \times \Psi(t - t_j)$ from Table IV for the appropriate value of h/d. Head the column with the opposite sign from that of B_j in the Table of processing history. Cross-add algebraically all the values under T_R, each J and each B to give T. The heat-penetration curve may now be plotted, on lethal-rate paper[7] if desired.

Calculation of F_C.—In the column headed L enter the lethal rate corresponding to each central temperature T and the value of z considered appropriate for the food in question. Values of L for temperatures up to 230° F for values of z in the range 14 to 21 are given in Table V. This is an extension of Table VI(a) of Part I, where values of L for temperatures above 230° F may be found. If M (and not z) is regarded as constant (see Part I) then calculate a suitable value of z from the formula $z = 710(T + 455)/M$, where T is the highest temperature attained at the centre. Having entered all the L values (correct to three places of decimals) add up the L column and multiply the total by f and by the common t interval, to give the F_C value of the process. An example should make this procedure clear.

Example 1.—A2 cans, $h/d = 1{\cdot}3$ (Part I, Table I); $f = 62$ min., $T_0 = 140°$ F. When steam is turned on the retort temperature jumps almost instantaneously to 210° F, is allowed to rise linearly to 240° F in 10 minutes, then allowed to rise to 250° F in a further 10 minutes. The retort is held at 250° F for 20 minutes and then allowed to fall to 240° F in 5 minutes. It is held at 240° F for 45 minutes (until the end of the process), when pressure cooling starts. It is assumed that the temperature outside the can falls instantaneously to that of the cooling water, 70° F. What is the F value of this process? Draw up the Table of processing history as shown (Table I); it is self-explanatory.

Table I

Processing history (Example 1)

θ	t	T_R	J	p	B
		140		0	
0		210	210 − 140 = 70		186 − 0 = 186
				(240 − 210) × 62/10 = 186	
10	0·161	240			62 − 186 = − 124
				(250 − 240) × 62/10 = 62	
20	0·323	250			0 − 62 = − 62
				0	
40	0·645	250			− 124 − 0 = − 124
				(240 − 250) × 62/5 = − 124	
45	0·726	240			0 − (− 124) = 124
				0	
90	1·452	240	70 − 240 = − 170		
		70			

Table II

Heat penetration (Example 1)

t	T_R	J_0^{70} −	B_0^{186} −	$B_{0\cdot161}^{-124}$ +	$B_{0\cdot323}^{-62}$ +	$B_{0\cdot645}^{-124}$ +	$B_{0\cdot726}^{124}$ −	$J_{1\cdot452}^{-170}$ +	T	L (z = 17·5)
0	210	70	0						140·0	
0·1	228·6	69·9	18·6						140·1	
0·2	242·4	67·3	37·0	4·8					142·9	
0·3	248·6	60·3	53·9	17·2					151·6	
0·4	250	51·2	68·8	28·1	4·8				162·9	
0·5	250	42·4	81·3	40·1	10·9				177·3	
0·6	250	34·4	91·3	49·4	16·7				190·4	0
0·7	243·2	27·7	99·7	57·0	21·9	6·8			201·5	0·002
0·8	240	22·2	106·2	63·2	26·2	19·2	9·2		211·0	0·006
0·9	240	17·7	111·6	68·3	29·7	31·1	21·5		218·3	0·016
1·0	240	14·1	115·7	72·3	32·7	41·7	33·2		223·7	0·032
1·1	240	11·3	119·0	75·5	35·0	50·8	43·5		227·5	0·052
1·2	240	9·0	121·8	78·0	36·8	58·1	52·2		229·9	0·071
1·3	240	7·1	123·9	80·1	38·3	64·1	59·3		232·2	0·096
1·4	240	5·7	125·6	81·8	39·4	68·9	65·2		233·6	0·116
1·5	70	4·5	126·9	83·1	40·4	72·9	69·8	170	235·2	0·143
1·6	70	3·6	128·0	84·1	41·1	76·0	73·5	168·5	234·6	0·132
1·7	70	2·9	128·9	84·9	41·7	78·4	76·5	156·4	223·1	0·029
1·8	70	2·3	129·5	85·6	42·2	80·4	78·7	136·2	203·9	0·002
1·9	70	1·8	130·0	86·1	42·6	82·0	80·7	113·9	182·1	0
									Total	0·697

$F_C = 0{\cdot}697 \times 62 \times 0{\cdot}1 = 4{\cdot}3$

Draw up the Table of heat penetration. This (Table II) was started at $t = 0$, to help in illustrating the method.

Column T_R: At $t = 0$, T_R jumps from 140° to 210° F and is entered as 210.

At $t = 0{\cdot}1$, $p = 186$, $T_R = 210 + 186(0{\cdot}1 - 0) = 228{\cdot}6$.
At $t = 0{\cdot}2$ (between $t = 0{\cdot}161$ and $0{\cdot}323$), $p = 62$, $T_R = 240 + 62(0{\cdot}2 - 0{\cdot}161) = 242{\cdot}4$.
At $t = 0{\cdot}3$, $T_R = 240 + 62(0{\cdot}3 - 0{\cdot}161) = 248{\cdot}6$.
At $t = 0{\cdot}4$, $p = 0$, $T_R = 250$.
At $t = 0{\cdot}7$ (between $t = 0{\cdot}645$ and $0{\cdot}726$), $p = -124$, $T_R = 250 - 124(0{\cdot}7 - 0{\cdot}645) = 243{\cdot}2$.

Column J_0^{70}: (Jump of + 70° at $t = 0$). Head the column minus. Use Table III under $h/d = 1{\cdot}3$.

At $t = 0$, enter $70 \times \Phi(0 - 0) = 70 \times 1 = 70$.
At $t = 0{\cdot}1$, ,, $70 \times \Phi(0{\cdot}1 - 0) = 70 \times 0{\cdot}999 = 69{\cdot}9$.
At $t = 0{\cdot}2$, ,, $70 \times \Phi(0{\cdot}2 - 0) = 70 \times 0{\cdot}962 = 67{\cdot}3$.

Column B_0^{186}: ($B = +186$ at $t = 0$). Head the column minus. Use Table IV under $h/d = 1{\cdot}3$.

At $t = 0$, enter $186 \times \Psi(0 - 0) = 186 \times 0 = 0$.
At $t = 0{\cdot}1$, ,, $186 \times \Psi(0{\cdot}1 - 0) = 186 \times 0{\cdot}1 = 18{\cdot}6$.
[for $t < 0{\cdot}12$ in Table IV, $\Psi(t) = t$]
At $t = 0{\cdot}2$, enter $186 \times \Psi(0{\cdot}2 - 0) = 186 \times 0{\cdot}199 = 37{\cdot}0$.

Column $B_{0\cdot161}^{-124}$: ($B = -124$ at $t = 0\cdot161$). Head the column plus.

At $t = 0\cdot2$, enter $124 \times \Psi(0\cdot2 - 0\cdot161) = 124 \times 0\cdot039 = 4\cdot8$.
At $t = 0\cdot3$, ,, $124 \times \Psi(0\cdot3 - 0\cdot161) = 124 \times 0\cdot139 = 17\cdot2$.
At $t = 1\cdot0$, ,, $124 \times \Psi(1\cdot0 - 0\cdot161) = 124 \times 0\cdot583 = 72\cdot3$.

Similarly the columns headed $B_{0\cdot323}^{-62}$, $B_{0\cdot645}^{-124}$, $B_{0\cdot726}^{124}$ and $J_{1\cdot452}^{-170}$ are entered. Cross-adding gives column T. It was decided that $z = 17\cdot5$ was appropriate for the product. $M = 28000$ was in fact selected, and z was found by the formula $z = 710(235 + 455)/28000 = 17\cdot5$, 235 being the highest temperature in column T.

Column L was filled in from Table V and Table VI(a) of Part I. The sum of the L values was multiplied by f (62) and by the common t interval (0·1) to give $F_C = 4\cdot3$. If this were considered an unnecessarily high F_C value, the effect of starting cooling five minutes earlier could be tested by amending the time of the second J and altering the corresponding column. It may be noted that if all the times of jumps and breaks were arranged to occur at multiples of the common t difference, each J and B column could be shifted up or down without need of recalculating the values. This would simplify the formulation of a process to attain a given F_C value.

Coming-up time of the retort

The initial rise of retort temperature to processing temperature is a special case of change of retort temperature that requires examination. It is useful to know the value of the coming-up time in terms of time at retort temperature. If the retort could have been raised instantaneously to processing temperature, it is required to know the time at this temperature that would be equivalent in F_C value to the actual coming-up time. In most cases this F_C value is negligibly small, and it is sufficiently accurate to find the time at processing temperature that would raise the temperature at the centre of the can to that reached at the end of the coming-up time.

The rise of temperature in the retort during the coming-up time is seldom rectilinear. It is more usually an exponential curve asymptotic to the temperature corresponding with the pressure of the steam supply, though, of course, the rise is cut off at processing temperature. If the pressure of the steam supply is very much greater than that corresponding with the processing temperature, the rise may be approximately rectilinear, or the inlet may be controlled to give a rectilinear rise, but usually the steam is turned full on and the retort allowed to rise as rapidly as possible to processing temperature, which may be only 10° or 20° F below that corresponding with the pressure of the steam supply. In this case the rise is exponential in terms of deficit from the asymptotic temperature [see equation (12) below] and the value of the coming-up time is greater than when the rise is rectilinear.

Let T_B represent the temperature corresponding with the pressure of the steam supply, i.e. the temperature that the retort would eventually reach if control were removed and the steam inlet left open. Let T_{R1} represent the processing temperature, T_0 the initial temperature in the cans, and assume the initial temperature in the retort also to be T_0. Let $(T_B - T_0)/(T_{R1} - T_0) = b$.

Let c minutes be the duration of the coming-up time of the retort, and r the fraction of c that may be added to the processing time W, so that c minutes coming-up and W minutes at T_{R1} is equivalent in sterilizing value to $rc + W$ minutes at T_{R1} with no coming-up time. Fig. 1 shows r plotted against c/f for various values of b. Linear rise of retort temperature during coming-up is represented by $b = \infty$. The formula by which these curves were calculated is derived in the Appendix. The curves refer to cans with $h/d = 0\cdot9$, and approximate corrections, which seldom exceed 10%, are given for cans of other relative dimensions.

Example 2.—Pressure of steam supply 20 lb./sq. in., equivalent to 259° F. Processing temperature 240° F. Initial temperature of cans (and of retort) 140° F. A2 cans, $f = 62$, $h/d = 1\cdot3$. Coming-up time of retort 20 minutes. What is the value of the coming-up time in terms of time at 240° F?

$b = (259 - 140)/(240 - 140) = 1\cdot19$; $c/f = 20/62 = 0\cdot323$
$r = 0\cdot735 - (1 - 0\cdot323)/40 = 0\cdot718$ (from Fig. 1)
Value of coming-up time $= 0\cdot718 \times 20 = 14\cdot4$ min.

If Fig. 1 is used for estimating the value of the coming-up time when c is large compared with f ($c/f > 2$), the value of r so found is likely to be too small. This may arise when the coming-up time is long and the cans are small, or if the method is applied to convection packs (which may be legitimate when the heat-penetration curve for a point near the bottom of the can is similar in form to that for the centre of a can heating by conduction). When c is

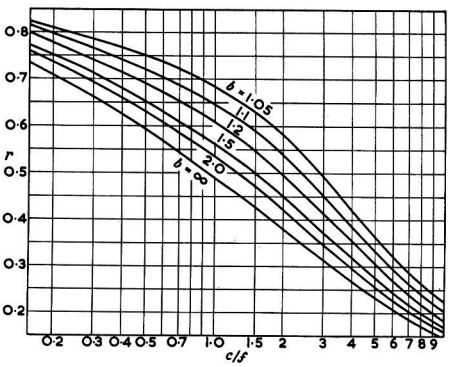

FIG. 1.—*Value of coming-up time of retort in terms of time at processing temperature*

The curves shown are for h/d = 0·9. Corrections for other values of h/d are as follows:

h/d	Subtract	h/d	Subtract
0·5	$(1 - c/f)/10$	0·7 and 1·3	$(1 - c/f)/40$
0·6 and 1·5	$(1 - c/f)/20$	0·8 and 1·1	$(1 - c/f)/80$

No correction is required for h/d when $c/f \geqslant 1{\cdot}0$

two or three times f, or more, the F_C value of the coming-up time may be appreciable and it is then not accurate to assume that times to reach the same central temperature are equivalent in F_C value. A correction may be applied to r.

After finding a preliminary value of r from Fig. 1, proceed as follows: calculate $rc/f - s$, where $s = \log 2{\cdot}04\ (T_R - T_0)/z$; find the corresponding value of u_H from Table III of Part I, and divide this by r to give the true u_H value of the coming-up time; from the same Table find the new value of $rc/f - s$ for this value of u_H and hence find the true value of r (r'). This procedure is justified in the Appendix.

Example 3.—As for Example 2 except that $f = 10$, $c = 30$, also $z = 17{\cdot}5$; $b = 1{\cdot}19$ as before, $c/f = 30/10 = 3{\cdot}0$. $r = 0{\cdot}41$ (from Fig. 1). Value of coming-up time $0{\cdot}41 \times 30 = 12{\cdot}3$ min. (uncorrected).

If it is desired to find the corrected value, proceed as follows: $s = \log (2{\cdot}04 \times 100/17{\cdot}5) = 1{\cdot}067$. $rc/f - s = 0{\cdot}41 \times 3{\cdot}0 - 1{\cdot}067 = 0{\cdot}16$. $u_H = 0{\cdot}038$ (from Table II of Part I), $u_H/r = 0{\cdot}038/0{\cdot}41 = 0{\cdot}093$. $r'c/f - s = 0{\cdot}36$ (from the same Table), $r' = (0{\cdot}36 + 1{\cdot}07)/3 = 0{\cdot}48$. Value of coming-up time $= 0{\cdot}48 \times 30 = 14{\cdot}4$ min.

Table III

Values of $\Phi(t)$ for various values of h/d

	h/d											
t	0·5	0·6	0·7	0·8	0·9	1·0	1·1	1·2	1·3	1·4	1·5	t
0·05	1	1	1	1	1	1	1	1	1	1	1	0·05
0·06	0·999	1	1	1	1	1	1	1	1	1	1	0·06
0·07	0·998	1	1	1	1	1	1	1	1	1	1	0·07
0·08	0·997	0·999	1	1	1	1	1	1	1	1	1	0·08
0·09	0·995	0·998	0·999	1	1	1	1	1	1	0·999	0·999	0·09
0·10	0·993	0·997	0·999	0·999	1	1	1	0·999	0·999	0·999	0·999	0·10
0·11	0·989	0·995	0·997	0·998	0·999	0·999	0·999	0·998	0·998	0·998	0·997	0·11
0·12	0·984	0·992	0·995	0·997	0·998	0·998	0·998	0·997	0·997	0·996	0·995	0·12
0·13	0·979	0·989	0·993	0·995	0·997	0·997	0·997	0·996	0·995	0·994	0·993	0·13
0·14	0·972	0·984	0·990	0·993	0·996	0·996	0·995	0·994	0·993	0·992	0·990	0·14
0·15	0·965	0·979	0·987	0·991	0·994	0·994	0·993	0·992	0·991	0·989	0·987	0·15
0·16	0·957	0·974	0·984	0·989	0·992	0·991	0·990	0·988	0·987	0·985	0·983	0·16
0·17	0·948	0·968	0·980	0·986	0·989	0·988	0·986	0·983	0·982	0·979	0·977	0·17
0·18	0·939	0·961	0·975	0·982	0·985	0·984	0·981	0·978	0·976	0·973	0·970	0·18
0·19	0·930	0·954	0·969	0·977	0·980	0·979	0·976	0·972	0·969	0·966	0·963	0·19
0·20	0·921	0·946	0·962	0·971	0·974	0·973	0·970	0·966	0·962	0·958	0·955	0·20
0·21	0·911	0·939	0·956	0·965	0·968	0·966	0·963	0·959	0·955	0·950	0·947	0·21
0·22	0·901	0·932	0·949	0·958	0·961	0·959	0·956	0·952	0·947	0·942	0·938	0·22
0·23	0·890	0·923	0·941	0·950	0·953	0·951	0·948	0·944	0·939	0·933	0·928	0·23
0·24	0·879	0·913	0·932	0·941	0·944	0·942	0·939	0·935	0·929	0·922	0·917	0·24

Table III (*contd.*)

Values of $\Phi(t)$ for various values of h/d

h/d												
	0·5	0·6	0·7	0·8	0·9	1·0	1·1	1·2	1·3	1·4	1·5	t
0·25	0·868	0·903	0·923	0·932	0·935	0·933	0·930	0·924	0·918	0·911	0·905	0·25
0·26	0·857	0·892	0·913	0·922	0·926	0·924	0·920	0·914	0·908	0·901	0·894	0·26
0·27	0·845	0·881	0·902	0·912	0·916	0·914	0·909	0·903	0·897	0·890	0·883	0·27
0·28	0·834	0·869	0·890	0·901	0·905	0·903	0·898	0·892	0·885	0·878	0·871	0·28
0·29	0·822	0·857	0·878	0·889	0·893	0·891	0·886	0·880	0·873	0·866	0·858	0·29
0·30	0·810	0·845	0·866	0·877	0·881	0·879	0·874	0·868	0·861	0·853	0·845	0·30
0·31	0·798	0·833	0·855	0·866	0·870	0·868	0·863	0·857	0·849	0·841	0·833	0·31
0·32	0·786	0·821	0·843	0·854	0·858	0·856	0·851	0·845	0·837	0·829	0·821	0·32
0·33	0·774	0·809	0·831	0·841	0·845	0·843	0·838	0·832	0·825	0·817	0·808	0·33
0·34	0·762	0·797	0·818	0·828	0·831	0·829	0·825	0·819	0·812	0·804	0·795	0·34
0·35	0·750	0·785	0·805	0·815	0·818	0·816	0·812	0·805	0·798	0·790	0·781	0·35
0·36	0·739	0·772	0·792	0·801	0·804	0·802	0·798	0·791	0·784	0·776	0·767	0·36
0·37	0·727	0·760	0·779	0·788	0·791	0·789	0·785	0·778	0·771	0·763	0·754	0·37
0·38	0·715	0·747	0·766	0·775	0·778	0·776	0·772	0·765	0·758	0·750	0·741	0·38
0·39	0·703	0·734	0·753	0·762	0·765	0·763	0·759	0·752	0·745	0·737	0·728	0·39
0·40	0·691	0·721	0·739	0·748	0·751	0·749	0·745	0·738	0·731	0·723	0·715	0·40
0·41	0·679	0·709	0·726	0·735	0·738	0·736	0·732	0·725	0·718	0·710	0·702	0·41
0·42	0·668	0·697	0·713	0·722	0·725	0·723	0·719	0·712	0·705	0·697	0·689	0·42
0·43	0·656	0·684	0·700	0·708	0·711	0·709	0·706	0·699	0·692	0·684	0·676	0·43
0·44	0·644	0·671	0·687	0·695	0·698	0·696	0·693	0·687	0·680	0·672	0·663	0·44
0·45	0·633	0·659	0·675	0·682	0·684	0·683	0·680	0·674	0·667	0·659	0·651	0·45
0·46	0·621	0·647	0·662	0·669	0·671	0·670	0·667	0·661	0·654	0·646	0·638	0·46
0·47	0·610	0·635	0·649	0·656	0·658	0·657	0·654	0·649	0·642	0·634	0·626	0·47
0·48	0·599	0·623	0·636	0·643	0·645	0·644	0·641	0·636	0·630	0·622	0·614	0·48
0·49	0·588	0·611	0·624	0·630	0·632	0·631	0·628	0·623	0·618	0·610	0·602	0·49
0·50	0·577	0·599	0·611	0·617	0·619	0·618	0·615	0·610	0·605	0·597	0·590	0·50
0·51	0·567	0·587	0·598	0·604	0·606	0·605	0·602	0·597	0·592	0·585	0·578	0·51
0·52	0·556	0·575	0·586	0·592	0·594	0·593	0·590	0·585	0·580	0·573	0·567	0·52
0·53	0·545	0·563	0·574	0·579	0·581	0·580	0·578	0·573	0·568	0·562	0·556	0·53
0·54	0·534	0·552	0·562	0·567	0·569	0·568	0·566	0·562	0·557	0·551	0·545	0·54
0·55	0·524	0·541	0·551	0·556	0·558	0·557	0·555	0·551	0·546	0·540	0·534	0·55
0·56	0·513	0·530	0·540	0·544	0·546	0·545	0·543	0·539	0·534	0·528	0·523	0·56
0·57	0·503	0·520	0·529	0·533	0·535	0·534	0·532	0·528	0·523	0·517	0·512	0·57
0·58	0·494	0·510	0·519	0·522	0·524	0·523	0·521	0·519	0·512	0·506	0·501	0·58
0·59	0·484	0·499	0·508	0·511	0·513	0·512	0·510	0·506	0·501	0·496	0·491	0·59
0·60	0·474	0·489	0·497	0·500	0·502	0·501	0·499	0·495	0·491	0·486	0·481	0·60
0·61	0·464	0·478	0·486	0·489	0·491	0·490	0·488	0·484	0·480	0·476	0·471	0·61
0·62	0·455	0·468	0·476	0·479	0·481	0·480	0·478	0·474	0·470	0·466	0·461	0·62
0·63	0·446	0·459	0·466	0·469	0·470	0·470	0·468	0·464	0·460	0·456	0·451	0·63
0·64	0·437	0·449	0·456	0·459	0·460	0·460	0·458	0·454	0·451	0·447	0·442	0·64
0·65	0·428	0·440	0·447	0·449	0·450	0·450	0·448	0·445	0·442	0·438	0·433	0·65
0·66	0·419	0·431	0·437	0·439	0·440	0·440	0·438	0·435	0·432	0·428	0·423	0·66
0·67	0·410	0·422	0·428	0·430	0·430	0·430	0·429	0·426	0·423	0·419	0·414	0·67
0·68	0·402	0·413	0·418	0·420	0·420	0·420	0·419	0·417	0·414	0·410	0·405	0·68
0·69	0·394	0·404	0·409	0·411	0·411	0·411	0·410	0·408	0·405	0·402	0·397	0·69
0·70	0·386	0·395	0·400	0·402	0·402	0·402	0·401	0·399	0·396	0·393	0·389	0·70
0·71	0·378	0·386	0·391	0·393	0·393	0·393	0·392	0·390	0·387	0·384	0·381	0·71
0·72	0·370	0·378	0·383	0·385	0·385	0·385	0·384	0·382	0·379	0·376	0·373	0·72
0·73	0·362	0·370	0·374	0·376	0·376	0·376	0·375	0·374	0·371	0·368	0·365	0·73
0·74	0·355	0·362	0·366	0·368	0·368	0·368	0·367	0·366	0·363	0·360	0·357	0·74
0·75	0·347	0·355	0·359	0·360	0·360	0·360	0·359	0·358	0·355	0·352	0·349	0·75
0·76	0·340	0·347	0·351	0·352	0·352	0·352	0·351	0·350	0·347	0·344	0·341	0·76
0·77	0·333	0·339	0·343	0·344	0·344	0·344	0·343	0·342	0·339	0·336	0·333	0·77
0·78	0·326	0·332	0·335	0·336	0·336	0·336	0·335	0·334	0·331	0·329	0·326	0·78
0·79	0·319	0·324	0·327	0·328	0·328	0·328	0·327	0·326	0·324	0·322	0·319	0·79
0·80	0·312	0·317	0·320	0·321	0·321	0·321	0·320	0·319	0·317	0·315	0·313	0·80
0·81	0·305	0·310	0·313	0·314	0·314	0·314	0·313	0·312	0·310	0·308	0·306	0·81
0·82	0·298	0·303	0·306	0·307	0·307	0·307	0·306	0·305	0·303	0·301	0·299	0·82
0·83	0·291	0·296	0·299	0·300	0·300	0·300	0·299	0·298	0·296	0·294	0·292	0·83
0·84	0·285	0·290	0·293	0·294	0·294	0·294	0·293	0·292	0·290	0·288	0·286	0·84
0·85	0·279	0·284	0·286	0·287	0·287	0·287	0·286	0·285	0·284	0·282	0·280	0·85
0·86	0·273	0·278	0·280	0·281	0·281	0·281	0·280	0·279	0·278	0·276	0·274	0·86
0·87	0·267	0·272	0·273	0·274	0·274	0·274	0·273	0·272	0·271	0·270	0·268	0·87
0·88	0·261	0·266	0·267	0·268	0·268	0·268	0·267	0·266	0·265	0·264	0·263	0·88
0·89	0·255	0·260	0·261	0·262	0·262	0·262	0·261	0·260	0·259	0·258	0·257	0·89
0·90	0·250	0·254	0·255	0·256	0·256	0·256	0·255	0·254	0·253	0·252	0·251	0·90
0·91	0·244	0·248	0·249	0·250	0·250	0·250	0·249	0·248	0·247	0·246	0·245	0·91
0·92	0·239	0·242	0·243	0·244	0·244	0·244	0·243	0·242	0·241	0·240	0·239	0·92
0·93	0·234	0·236	0·237	0·238	0·238	0·238	0·237	0·236	0·235	0·234	0·233	0·93

Table III (*contd.*)

Values of $\Phi(t)$ for various values of h/d

	h/d											
t	0·5	0·6	0·7	0·8	0·9	1·0	1·1	1·2	1·3	1·4	1·5	t
0·94	0·229	0·231	0·232	0·233	0·233	0·233	0·232	0·231	0·230	0·229	0·228	0·94
0·95	0·224	0·226	0·227	0·228	0·228	0·228	0·227	0·226	0·225	0·224	0·223	0·95
0·96	0·219	0·221	0·222	0·223	0·223	0·223	0·222	0·221	0·220	0·219	0·218	0·96
0·97	0·214	0·216	0·217	0·218	0·218	0·218	0·217	0·216	0·215	0·214	0·213	0·97
0·98	0·209	0·211	0·212	0·213	0·213	0·213	0·213	0·212	0·211	0·210	0·209	0·98
0·99	0·204	0·206	0·207	0·208	0·208	0·208	0·208	0·207	0·206	0·205	0·204	0·99
1·00	0·200	0·202	0·203	0·204	0·204	0·204	0·204	0·203	0·202	0·201	0·200	1·00
1·01	0·195	0·197	0·198	0·199	0·199	0·199	0·199	0·198	0·197	0·196	0·195	1·01
1·02	0·191	0·193	0·194	0·195	0·195	0·195	0·195	0·194	0·193	0·192	0·191	1·02
1·03	0·187	0·188	0·189	0·190	0·190	0·190	0·190	0·189	0·188	0·187	0·186	1·03
1·04	0·183	0·184	0·185	0·186	0·186	0·186	0·186	0·185	0·184	0·183	0·182	1·04
1·05	0·179	0·180	0·181	0·182	0·182	0·182	0·182	0·181	0·180	0·179	0·178	1·05
1·06	0·175	0·176	0·177	0·178	0·178	0·178	0·178	0·177	0·176	0·175	0·174	1·06
1·07	0·171	0·172	0·173	0·174	0·174	0·174	0·174	0·173	0·172	0·171	0·170	1·07
1·08	0·169	0·168	0·169	0·170	0·170	0·170	0·170	0·169	0·168	0·168	0·167	1·08
1·09	0·163	0·164	0·165	0·166	0·166	0·166	0·166	0·165	0·164	0·164	0·163	1·09
1·10	0·160	0·161	0·162	0·162	0·162	0·162	0·162	0·161	0·161	0·161	0·160	1·10
1·11	0·156	0·157	0·158	0·158	0·158	0·158	0·158	0·157	0·157	0·157	0·156	1·11
1·12	0·153	0·154	0·155	0·155	0·155	0·155	0·155	0·154	0·154	0·154	0·153	1·12
1·13	0·150	0·150	0·151	0·151	0·151	0·151	0·151	0·150	0·150	0·150	0·149	1·13
1·14	0·147	0·147	0·148	0·148	0·148	0·148	0·148	0·147	0·147	0·147	0·146	0·14
1·15	0·143	0·143	0·144	0·144	0·144	0·144	0·144	0·143	0·143	0·143	0·143	1·15
1·16	0·140	0·140	0·141	0·141	0·141	0·141	0·141	0·140	0·140	0·140	0·140	1·16
1·17	0·137	0·137	0·138	0·138	0·138	0·138	0·138	0·137	0·137	0·137	0·137	1·17
1·18	0·134	0·134	0·135	0·135	0·135	0·135	0·135	0·134	0·134	0·134	0·134	1·18
1·19	0·131	0·131	0·132	0·132	0·132	0·132	0·132	0·131	0·131	0·131	0·131	1·19
1·20	0·128	0·128	0·129	0·129	0·129	0·129	0·129	0·128	0·128	0·128	0·128	1·20
1·21	0·125	0·125	0·126	0·126	0·126	0·126	0·126	0·125	0·125	0·125	0·125	1·21
1·22	0·122	0·123	0·123	0·123	0·123	0·123	0·122	0·122	0·122	0·122	0·122	1·22
1·23	0·119	0·120	0·120	0·120	0·120	0·120	0·120	0·119	0·119	0·119	0·119	1·23
1·24	0·117	0·117	0·117	0·117	0·117	0·117	0·117	0·117	0·117	0·117	0·117	1·24

Values of $\Phi(t)$ for all values of h/d

t	$\Phi(t)$	t	$\Phi(t)$	t	$\Phi(t)$	t	$\Phi(t)$		$\Phi(t)$	t	$\Phi(t)$
1·24	0·117	1·40	0·081	1·65	0·046	1·90	0·026	2·3	0·010	2·9	0·003
1·26	0·112	1·45	0·072	1·70	0·041	1·95	0·023	2·4	0·008	3·0	0·002
1·28	0·107	1·50	0·064	1·75	0·036	2·00	0·020	2·5	0·006	3·1	0·002
1·30	0·102	1·55	0·057	1·80	0·032	2·1	0·016	2·6	0·005	3·2	0·001
1·35	0·091	1·60	0·051	1·85	0·029	2·2	0·013	2·7	0·004	3·6	0·001
1·40	0·081	1·65	0·046	1·90	0·026	2·3	0·010	2·8	0·003	3·7	0·000

Table IV

Values of $\Psi(t)$ for various values of h/d

	h/d											
	0·5	0·6	0·7	0·8	0·9	1·0	1·1	1·2	1·3	1·4	1·5	t
0·12	0·120	0·120	0·120	0·120	0·120	0·120	0·120	0·120	0·120	0·120	0·120	0·12
0·13	0·129	0·130	0·130	0·130	0·130	0·130	0·130	0·130	0·130	0·130	0·130	0·13
0·14	0·139	0·140	0·140	0·140	0·140	0·140	0·140	0·140	0·140	0·140	0·140	0·14
0·15	0·149	0·149	0·150	0·150	0·150	0·150	0·150	0·150	0·150	0·150	0·150	0·15
0·16	0·159	0·159	0·160	0·160	0·160	0·160	0·160	0·160	0·160	0·160	0·159	0·16
0·17	0·168	0·169	0·169	0·170	0·170	0·170	0·170	0·170	0·169	0·169	0·169	0·17
0·18	0·177	0·179	0·179	0·179	0·180	0·180	0·179	0·179	0·179	0·179	0·179	0·18
0·19	0·186	0·188	0·189	0·189	0·189	0·189	0·189	0·189	0·189	0·189	0·189	0·19
0·20	0·196	0·198	0·199	0·199	0·199	0·199	0·199	0·199	0·199	0·198	0·198	0·20
0·21	0·205	0·207	0·208	0·209	0·209	0·209	0·209	0·209	0·209	0·208	0·208	0·21
0·22	0·214	0·216	0·218	0·219	0·219	0·219	0·219	0·219	0·219	0·218	0·218	0·22
0·23	0·223	0·225	0·227	0·228	0·228	0·228	0·228	0·228	0·228	0·227	0·227	0·23
0·24	0·232	0·234	0·236	0·237	0·238	0·238	0·238	0·238	0·237	0·236	0·236	0·24
0·25	0·241	0·244	0·246	0·247	0·247	0·247	0·247	0·247	0·246	0·245	0·245	0·25
0·26	0·249	0·253	0·255	0·256	0·256	0·256	0·256	0·255	0·255	0·254	0·254	0·26
0·27	0·258	0·262	0·264	0·265	0·265	0·265	0·265	0·264	0·264	0·263	0·263	0·27
0·28	0·266	0·271	0·273	0·274	0·274	0·274	0·274	0·273	0·273	0·272	0·271	0·28

Table IV (*contd.*)

Values of $\Psi(t)$ for various values of h/d

	h/d											
t	0·5	0·6	0·7	0·8	0·9	1·0	1·1	1·2	1·3	1·4	1·5	
0·29	0·275	0·280	0·282	0·283	0·283	0·283	0·283	0·282	0·282	0·281	0·280	0·29
0·30	0·283	0·288	0·291	0·292	0·292	0·292	0·292	0·291	0·290	0·289	0·289	0·30
0·31	0·291	0·296	0·299	0·301	0·301	0·301	0·301	0·300	0·299	0·298	0·298	0·31
0·32	0·299	0·304	0·308	0·310	0·310	0·310	0·310	0·309	0·308	0·307	0·307	0·32
0·33	0·306	0·312	0·316	0·318	0·318	0·318	0·318	0·317	0·316	0·315	0·315	0·33
0·34	0·314	0·320	0·324	0·326	0·327	0·327	0·326	0·325	0·324	0·323	0·323	0·34
0·35	0·322	0·328	0·332	0·334	0·335	0·335	0·334	0·333	0·332	0·331	0·331	0·35
0·36	0·329	0·336	0·340	0·342	0·343	0·343	0·342	0·341	0·340	0·339	0·338	0·36
0·37	0·337	0·344	0·348	0·350	0·351	0·351	0·350	0·349	0·348	0·347	0·346	0·37
0·38	0·344	0·351	0·356	0·358	0·359	0·359	0·358	0·357	0·356	0·354	0·353	0·38
0·39	0·351	0·359	0·364	0·366	0·367	0·367	0·366	0·364	0·363	0·361	0·360	0·39
0·40	0·358	0·366	0·371	0·373	0·374	0·374	0·373	0·371	0·370	0·368	0·367	0·40
0·41	0·365	0·373	0·378	0·381	0·382	0·382	0·381	0·379	0·378	0·376	0·375	0·41
0·42	0·371	0·380	0·385	0·388	0·389	0·389	0·388	0·386	0·385	0·383	0·382	0·42
0·43	0·378	0·387	0·392	0·395	0·396	0·396	0·395	0·393	0·392	0·390	0·389	0·43
0·44	0·385	0·394	0·399	0·402	0·403	0·403	0·402	0·400	0·399	0·397	0·396	0·44
0·45	0·391	0·400	0·406	0·409	0·410	0·410	0·409	0·407	0·406	0·404	0·402	0·45
0·46	0·397	0·407	0·413	0·416	0·417	0·417	0·416	0·414	0·413	0·410	0·408	0·46
0·47	0·403	0·413	0·420	0·423	0·424	0·424	0·423	0·421	0·419	0·416	0·414	0·47
0·48	0·409	0·419	0·426	0·429	0·430	0·430	0·429	0·427	0·425	0·422	0·420	0·48
0·49	0·415	0·426	0·433	0·436	0·437	0·436	0·435	0·433	0·431	0·428	0·426	0·49
0·50	0·421	0·432	0·439	0·442	0·443	0·442	0·441	0·439	0·437	0·434	0·432	0·50
0·51	0·427	0·438	0·445	0·448	0·449	0·448	0·447	0·445	0·443	0·440	0·438	0·51
0·52	0·432	0·443	0·450	0·454	0·455	0·454	0·453	0·451	0·449	0·446	0·444	0·52
0·53	0·438	0·449	0·456	0·460	0·461	0·460	0·459	0·457	0·455	0·452	0·450	0·53
0·54	0·443	0·455	0·462	0·465	0·466	0·465	0·464	0·462	0·460	0·457	0·455	0·54
0·55	0·449	0·461	0·468	0·471	0·472	0·471	0·470	0·468	0·466	0·463	0·460	0·55
0·56	0·454	0·466	0·473	0·476	0·477	0·476	0·475	0·473	0·471	0·468	0·465	0·56
0·57	0·459	0·471	0·479	0·482	0·483	0·482	0·481	0·479	0·476	0·473	0·470	0·57
0·58	0·464	0·476	0·484	0·487	0·488	0·487	0·486	0·484	0·481	0·478	0·475	0·58
0·59	0·469	0·481	0·489	0·492	0·493	0·492	0·491	0·489	0·486	0·483	0·480	0·59
0·60	0·474	0·486	0·494	0·497	0·498	0·497	0·496	0·494	0·491	0·488	0·485	0·60
0·61	0·479	0·491	0·499	0·502	0·503	0·502	0·501	0·499	0·496	0·493	0·490	0·61
0·62	0·483	0·496	0·504	0·507	0·508	0·507	0·506	0·504	0·501	0·498	0·495	0·62
0·63	0·488	0·501	0·509	0·512	0·513	0·512	0·510	0·508	0·505	0·502	0·499	0·63
0·64	0·492	0·505	0·513	0·517	0·518	0·517	0·515	0·513	0·510	0·507	0·504	0·64
0·65	0·496	0·510	0·518	0·522	0·523	0·522	0·520	0·518	0·515	0·512	0·508	0·65
0·66	0·500	0·514	0·522	0·526	0·527	0·526	0·524	0·522	0·519	0·516	0·513	0·66
0·67	0·504	0·518	0·526	0·530	0·532	0·531	0·529	0·527	0·524	0·520	0·517	0·67
0·68	0·508	0·522	0·530	0·534	0·536	0·535	0·533	0·531	0·528	0·524	0·521	0·68
0·69	0·512	0·526	0·534	0·538	0·540	0·539	0·537	0·535	0·532	0·528	0·525	0·69
0·70	0·516	0·530	0·538	0·542	0·544	0·543	0·541	0·539	0·536	0·532	0·529	0·70
0·71	0·519	0·534	0·542	0·546	0·548	0·547	0·545	0·543	0·540	0·536	0·533	0·71
0·72	0·523	0·538	0·546	0·549	0·551	0·550	0·548	0·546	0·543	0·539	0·536	0·72
0·73	0·527	0·542	0·550	0·553	0·555	0·554	0·552	0·550	0·547	0·543	0·540	0·73
0·74	0·531	0·546	0·554	0·557	0·559	0·558	0·556	0·554	0·551	0·547	0·544	0·74
0·75	0·534	0·549	0·557	0·560	0·562	0·561	0·559	0·557	0·554	0·550	0·547	0·75
0·76	0·538	0·553	0·561	0·564	0·566	0·565	0·563	0·561	0·558	0·554	0·551	0·76
0·77	0·541	0·557	0·565	0·567	0·569	0·568	0·566	0·564	0·561	0·557	0·554	0·77
0·78	0·545	0·560	0·568	0·571	0·573	0·572	0·570	0·568	0·565	0·561	0·558	0·78
0·79	0·548	0·563	0·571	0·575	0·577	0·576	0·574	0·571	0·568	0·564	0·561	0·79
0·80	0·551	0·566	0·574	0·578	0·580	0·579	0·577	0·574	0·571	0·567	0·564	0·80
0·81	0·554	0·569	0·577	0·581	0·583	0·582	0·580	0·577	0·574	0·570	0·567	0·81
0·82	0·557	0·572	0·580	0·584	0·586	0·585	0·583	0·580	0·577	0·573	0·570	0·82
0·83	0·560	0·575	0·583	0·587	0·589	0·588	0·586	0·583	0·580	0·576	0·573	0·83
0·84	0·563	0·578	0·586	0·590	0·592	0·591	0·589	0·586	0·583	0·579	0·576	0·84
0·85	0·566	0·581	0·590	0·593	0·595	0·594	0·592	0·589	0·586	0·582	0·579	0·85
0·86	0·569	0·584	0·593	0·596	0·598	0·597	0·595	0·592	0·589	0·585	0·582	0·86
0·87	0·572	0·587	0·596	0·599	0·601	0·600	0·598	0·595	0·592	0·588	0·585	0·87
0·88	0·574	0·589	0·598	0·601	0·603	0·602	0·601	0·598	0·595	0·591	0·587	0·88
0·89	0·577	0·592	0·601	0·604	0·606	0·605	0·604	0·601	0·598	0·594	0·590	0·89
0·90	0·579	0·594	0·603	0·606	0·608	0·607	0·606	0·603	0·600	0·596	0·592	0·90
0·91	0·582	0·597	0·606	0·609	0·611	0·610	0·609	0·606	0·603	0·599	0·595	0·91
0·92	0·584	0·599	0·608	0·611	0·613	0·612	0·611	0·608	0·605	0·601	0·597	0·92
0·93	0·587	0·602	0·611	0·614	0·616	0·615	0·613	0·610	0·607	0·603	0·599	0·93
0·94	0·589	0·604	0·613	0·616	0·618	0·617	0·615	0·612	0·609	0·605	0·601	0·94
0·95	0·592	0·607	0·616	0·619	0·621	0·620	0·618	0·615	0·612	0·608	0·604	0·95
0·96	0·594	0·609	0·618	0·621	0·623	0·622	0·620	0·617	0·614	0·610	0·606	0·96
0·97	0·596	0·611	0·620	0·623	0·625	0·624	0·622	0·619	0·616	0·612	0·608	0·97

Table IV (*contd.*)

Values of $\Psi(t)$ for various values of h/d

	h/d											
t	0·5	0·6	0·7	0·8	0·9	1·0	1·1	1·2	1·3	1·4	1·5	
0·98	0·598	0·613	0·622	0·625	0·627	0·626	0·624	0·621	0·618	0·614	0·610	0·98
0·99	0·600	0·615	0·624	0·627	0·629	0·628	0·626	0·623	0·620	0·616	0·612	0·99
1·00	0·602	0·617	0·626	0·629	0·631	0·630	0·628	0·625	0·622	0·618	0·614	1·00
1·01	0·604	0·619	0·628	0·631	0·633	0·632	0·630	0·627	0·624	0·620	0·616	1·01
1·02	0·605	0·621	0·629	0·632	0·634	0·633	0·631	0·628	0·625	0·621	0·617	1·02
1·03	0·607	0·623	0·631	0·634	0·636	0·635	0·633	0·630	0·627	0·623	0·619	1·03
1·04	0·609	0·624	0·633	0·636	0·638	0·637	0·635	0·632	0·629	0·625	0·621	1·04
1·05	0·611	0·626	0·635	0·638	0·640	0·639	0·637	0·634	0·631	0·627	0·623	1·05
1·06	0·613	0·628	0·637	0·640	0·642	0·641	0·639	0·636	0·633	0·629	0·625	1·06
1·07	0·615	0·630	0·639	0·642	0·644	0·643	0·641	0·638	0·635	0·631	0·627	1·07
1·08	0·616	0·631	0·640	0·643	0·645	0·644	0·642	0·639	0·636	0·632	0·628	1·08
1·09	0·618	0·633	0·642	0·645	0·647	0·646	0·644	0·641	0·638	0·634	0·630	1·09
1·10	0·620	0·635	0·644	0·647	0·649	0·648	0·646	0·643	0·640	0·636	0·632	1·10
1·11	0·622	0·637	0·646	0·649	0·651	0·650	0·648	0·645	0·642	0·638	0·634	1·11
1·12	0·623	0·638	0·647	0·650	0·652	0·651	0·649	0·646	0·643	0·639	0·635	1·12
1·13	0·625	0·640	0·649	0·652	0·654	0·653	0·651	0·648	0·645	0·641	0·637	1·13
1·14	0·626	0·641	0·650	0·653	0·655	0·654	0·652	0·649	0·646	0·642	0·638	1·14
1·15	0·627	0·643	0·652	0·655	0·657	0·656	0·654	0·651	0·648	0·644	0·640	1·15
1·16	0·628	0·644	0·653	0·656	0·658	0·657	0·655	0·652	0·649	0·645	0·641	1·16

Values of $\Psi(t)$ for $h/d = 0·9$

t	$\Psi(t)$	t	$\Psi(t)$	t	$\Psi(t)$	t	$\Psi(t)$	t	$\Psi(t)$
1·16	0·658	1·30	0·675	1·65	0·700	2·0	0·710	2·7	0·717
1·18	0·661	1·35	0·680	1·70	0·702	2·1	0·712	2·8	0·718
1·20	0·664	1·40	0·684	1·75	0·704	2·2	0·714	2·9	0·718
1·22	0·666	1·45	0·688	1·80	0·705	2·3	0·715	3·0	0·718
1·24	0·669	1·50	0·691	1·85	0·707	2·4	0·716	3·1	0·718
1·26	0·671	1·55	0·694	1·90	0·708	2·5	0·716	3·2	0·719
1·28	0·673	1·60	0·697	1·95	0·709	2·6	0·717	> 3·2	0·719
1·30	0·675	1·65	0·700	2·00	0·710	2·7	0·717		

Correction for other values of h/d

h/d	Subtract
0·5	0·030
0·6	0·014
0·7	0·005
0·8	0·002
1·0	0·001
1·1	0·003
1·2	0·006
1·3	0·009
1·4	0·013
1·5	0·017

Table V

Values of L for various values of z

T	z	T	z		T	z			
	21		20	21		18	19	20	21
180	0	184	0·001	0·001	188	0	0·001	0·001	0·001
181	0·001	185	0·001	0·001	189	0	0·001	0·001	0·001
182	0·001	186	0·001	0·001	190	0	0·001	0·001	0·001
183	0·001	187	0·001	0·001	191	0·001	0·001	0·001	0·002

	z								
T	14	15	16	17	18	19	20	21	T
192	0	0	0	0	0·001	0·001	0·001	0·002	192
193	0	0	0	0	0·001	0·001	0·001	0·002	193
194	0	0	0	0·001	0·001	0·001	0·002	0·002	194
195	0	0	0	0·001	0·001	0·001	0·002	0·002	195
196	0	0	0	0·001	0·001	0·001	0·002	0·003	196
197	0	0	0	0·001	0·001	0·002	0·002	0·003	197
198	0	0	0·001	0·001	0·001	0·002	0·003	0·003	198
199	0	0	0·001	0·001	0·001	0·002	0·003	0·004	199
200	0	0	0·001	0·001	0·002	0·002	0·003	0·004	200
201	0	0·001	0·001	0·001	0·002	0·003	0·004	0·005	201
202	0	0·001	0·001	0·002	0·002	0·003	0·004	0·005	202
203	0	0·001	0·001	0·002	0·002	0·003	0·004	0·006	203
204	0·001	0·001	0·001	0·002	0·003	0·004	0·005	0·006	204
205	0·001	0·001	0·002	0·002	0·003	0·004	0·006	0·007	205
206	0·001	0·001	0·002	0·003	0·004	0·005	0·006	0·008	206

Table V (*contd.*)

Values of L for various values of z

	z								
T	14	15	16	17	18	19	20	21	T
207	0·001	0·001	0·002	0·003	0·004	0·005	0·007	0·009	207
208	0·001	0·002	0·002	0·003	0·005	0·006	0·008	0·010	208
209	0·001	0·002	0·003	0·004	0·005	0·007	0·009	0·011	209
210	0·001	0·002	0·003	0·004	0·006	0·008	0·010	0·012	210
211	0·002	0·003	0·004	0·005	0·007	0·009	0·011	0·014	211
212	0·002	0·003	0·004	0·006	0·008	0·010	0·013	0·016	212
213	0·002	0·003	0·005	0·007	0·009	0·011	0·014	0·017	213
214	0·003	0·004	0·006	0·008	0·010	0·013	0·016	0·019	214
215	0·003	0·005	0·006	0·009	0·011	0·014	0·018	0·022	215
216	0·004	0·005	0·007	0·010	0·013	0·016	0·020	0·024	216
217	0·004	0·006	0·009	0·011	0·015	0·018	0·022	0·027	217
218	0·005	0·007	0·010	0·013	0·017	0·021	0·025	0·030	218
219	0·006	0·009	0·012	0·015	0·019	0·023	0·028	0·033	219
220	0·007	0·010	0·013	0·017	0·022	0·026	0·032	0·037	220
221	0·008	0·012	0·015	0·020	0·024	0·030	0·035	0·042	221
222	0·010	0·014	0·018	0·023	0·028	0·034	0·040	0·046	222
223	0·012	0·016	0·021	0·026	0·032	0·038	0·045	0·052	223
224	0·014	0·018	0·024	0·030	0·036	0·043	0·050	0·058	224
225	0·016	0·022	0·027	0·034	0·041	0·048	0·056	0·064	225
226	0·019	0·025	0·032	0·039	0·046	0·055	0·063	0·072	226
227	0·023	0·029	0·037	0·044	0·053	0·062	0·071	0·080	227
228	0·027	0·034	0·042	0·051	0·060	0·070	0·079	0·090	228
229	0·032	0·040	0·049	0·058	0·068	0·078	0·089	0·100	229
230	0·037	0·046	0·056	0·067	0·077	0·089	0·100	0·112	230

For temperatures above 230° F, see Table VI(*a*) of Part I or use the formula $\log L = -(250 - T)/z$

Appendix

Let the temperature T at the centre of a can heating by conduction in a retort at constant temperature T_{R1} be defined by

$$(T_{R1} - T)/(T_{R1} - T_0) = \Phi(t) \quad (t = 0,\ T = T_0) \qquad . \quad . \quad . \quad (1)$$

where t is the time of heating in units of f minutes and f is as defined in Part I. If the time of heating is θ minutes, then $t = \theta/f$. $\Phi(t)$ is of the form[1]

$$\sum_{n=1}^{\infty} a_n \exp(-b_n t) \Big/ \sum_{n=1}^{\infty} a_n$$

Now if the retort temperature T_R is varied during the process, let the value of T_R at time t be defined by

$$(T_R - T_0)/(T_B - T_0) = \Theta(t) \quad (t = 0,\ T_R = T_0) \qquad . \quad . \quad . \quad (2)$$

where T_B is a constant reference temperature.

It is convenient to assume that the whole can is at uniform temperature T_0 at time $t = 0$. and also that the retort temperature is initially at the same temperature T_0.

Then, by Duhamel's theorem,[1] from (1) and (2)

$$\begin{aligned}(T - T_0)/(T_B - T_0) &= \int_0^t \Theta(\lambda)\frac{\partial}{\partial t}\{1 - \Phi(t - \lambda)\}d\lambda \\ &= \int_0^t \Theta(\lambda)\Phi'(t - \lambda)d\lambda = \Big[\Theta(\lambda)\Phi(t - \lambda)\Big]_{\lambda=0}^{t} - \int_0^t \Phi(t - \lambda)\Theta'(\lambda)d\lambda \\ &= \Theta(t) - \int_0^t \Phi(t - \lambda)\Theta'(\lambda)d\lambda \qquad [\text{since } \Theta(0) = 0 \text{ and } \Phi(0) = 1]\end{aligned}$$

Therefore, substituting for $\Theta(t)$ from (2),

$$T = T_R - (T_B - T_0)\int_0^t \Phi(t - \lambda)\Theta'(\lambda)d\lambda \qquad . \quad . \quad . \quad . \quad (3)$$

where T is the temperature at the centre of the can at time t.

Two cases for the function $\Theta(t)$ will be considered: (i) When changes in retort temperature are always rectilinear or instantaneous, i.e. the graph of retort temperature against time

is a series of straight lines. This is a condition of varying processing temperature that may be treated quite simply, and, apart from the initial coming-up of the retort, is the condition that ensues in practice, at least approximately. (ii) When the initial rise of retort temperature, measured as deficit from an asymptotic temperature (corresponding with the pressure of the steam supply), is exponential.

Although a linear rise in retort temperature is a special case of exponential rise, it is convenient to consider linear rise first.

Rectilinear changes in retort temperature.—Let the breaks in slope of the series of straight lines representing the plot of retort temperature against time occur at times t_0 (= 0), t_1, t_2, . . . t_n . . . , and assume for the moment that no instantaneous changes occur. From $t = -\infty$ to $t = t_0$ the retort temperature is assumed to be T_0.

If T_R is retort temperature at time $t(t_n < t < t_{n+1})$, then

$$T_R = T_0 + p_0 t_1 + p_1(t_2 - t_1) + \ldots + p_i(t_{i+1} - t_i) + \ldots + p_n(t - t_n) \quad . \quad (4)$$

where p_i is a constant defining the change of retort temperature in unit time during the time interval t_i to t_{i+1}.

$$\text{i.e. } p_i = (T_{R_{i+1}} - T_{R_i})/(t_{i+1} - t_i) \quad . \quad . \quad . \quad . \quad . \quad (5)$$

Equation (4) may be rearranged to give

$$T_R = T_0 + p_0 t + \sum_{i=1}^{n} (p_i - p_{i-1})(t - t_i) \quad . \quad . \quad . \quad . \quad (6)$$

As, by hypothesis, $T_R = T_0$ from $t = -\infty$ to $t = t_0 (= 0)$, then $p_{-1} = 0$ and (6) may be written

$$T_R = T_0 + \sum_{i=0}^{n} (p_i - p_{i-1})(t - t_i) \quad . \quad . \quad . \quad . \quad . \quad (7)$$

From (2) and (7) we have

$$\Theta(t) = \frac{1}{T_B - T_0} \sum_{i=0}^{n} (p_i - p_{i-1})(t - t_i)$$

Thus (3) may be written

$$T = T_R - \sum_{i=0}^{n} (p_i - p_{i-1}) \int_0^{t-t_i} \Phi(t - t_i - \lambda)\, d\lambda$$

or

$$T = T_R - \sum_{i=0}^{n} (p_i - p_{i-1}) \int_0^{t-t_i} \Phi(x)\, dx \quad . \quad . \quad . \quad . \quad . \quad (8)$$

Putting $\int_0^t \Phi(x)dx = \Psi(t)$, and $(p_i - p_{i-1}) = B_i$, (8) becomes

$$T = T_R - \sum_{i=0}^{n} B_i \Psi(t - t_i) \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (9)$$

Values of $\Phi(t)$ and $\Psi(t)$, which vary with the relative dimensions of the can, are given in Tables III and IV respectively.

Sometimes there may be an instantaneous or almost instantaneous change in retort temperature, as when a can passes from one unit of an automatic cooker to another, or into the cooler, or when pressure cooling starts in a retort.

A jump in retort temperature at time t_i is equivalent to interposing a term in (9) at time t_i of very short duration δt. Thus, if four consecutive breaks in the curve occur at t_{i-1}, t_i, $(t_i + \delta t)$ and t_{i+1}, let the three slopes be p_{i-1} between t_{i-1} and t_i, p_j between t_i and $(t_i + \delta t)$, p_i between $(t_i + \delta t)$ and t_{i+1}. The corresponding terms in (9) are

$$(p_j - p_{i-1})\Psi(t - t_i) + (p_i - p_j)\Psi(t - t_i - \delta t) \quad . \quad . \quad . \quad (10)$$

or $p_j\{\Psi(t - t_i) - \Psi(t - t_i - \delta t)\} + p_i\Psi(t - t_i - \delta t) - p_{i-1}\Psi(t - t_i)$.

But, in the limit $\delta t \to 0$

$$\Psi(t - t_i) - \Psi(t - t_i - \delta t) = \{\Phi(t - t_i)\}\delta t$$

and

$$\Psi(t - t_i)/\Psi(t - t_i - \delta t) = 1;$$

also, from (5) $$p_j = (T_{R_{t+\delta t}} - T_{R_{t_i}})/(t_i + \delta t - t_i) = J_i/\delta t$$

where J_i is the magnitude of the jump in retort temperature at time t_i. The terms in (10) now become $J_i\Phi(t - t_i) + B_i\Psi(t - t_i)$. It follows that equation (9) may be written, so as to include the effects of all jumps and breaks in the retort temperature ' plot ' up to time t, as

$$T = T_R - \sum_{i=0}^{n} \{J_i\Phi(t - t_i) + B_i\Psi(t - t_i)\} \quad . \quad . \quad . \quad . \quad (11)$$

where T is the temperature at the centre of the can at time t and T_R is the retort temperature at time t ($t_n < t < t_{n+1}$).

Exponential rise of retort temperature during the coming-up time.—Let the temperature T_R in the retort at time t be defined by

$$(T_B - T_R)/(T_B - T_0) = e^{-gt} \quad (t = 0,\ T_R = T_0) \quad . \quad . \quad . \quad (12)$$

where T_B is the asymptotic value of T_R (and is the temperature corresponding with the pressure of the steam supply) and g is a constant. Identifying T_B with T_B of equation (2), we have, from (2) and (12), $\Theta(t) = 1 - e^{-gt}$, and $\Theta'(t) = ge^{-gt}$. Hence (3) becomes

$$T = T_R - g(T_B - T_0)\int_0^t \Phi(t - \lambda)\, e^{-g\lambda}\, d\lambda \quad . \quad . \quad . \quad . \quad (13)$$

Now let the coming-up time be c minutes, at which time the retort temperature becomes constant at $T_R = T_{R1}$. From (13), putting $t = c/f$, the temperature T_1 at the centre of the can at the end of the coming-up time is given by

$$T_1 = T_{R1} - g(T_B - T_0)\int_0^{c/f} \Phi(c/f - \lambda)\, e^{-g\lambda}\, d\lambda$$

or

$$\frac{T_{R1} - T_1}{T_{R1} - T_0} = g\frac{T_B - T_0}{T_{R1} - T_0}\int_0^{c/f} \Phi(c/f - \lambda)\, e^{-g\lambda}\, d\lambda \quad . \quad . \quad . \quad (14)$$

From (12), $g = -f/c \ln \dfrac{T_B - T_{R1}}{T_B - T_0}$

Putting $\dfrac{T_B - T_0}{T_{R1} - T_0} = b$, (14) becomes

$$\frac{T_{R1} - T_1}{T_{R1} - T_0} = \frac{fb}{c} \ln \frac{b - 1}{b} \int_{c/f}^{0} \left(\frac{b - 1}{b}\right)^{f\lambda/c} \Phi(c/f - \lambda)\, d\lambda \quad . \quad . \quad . \quad (15)$$

If the retort temperature were raised instantaneously from T_0 to T_{R1}, then, from (1) the time θ_1 for the temperature at the centre of the can to reach T_1 would be given by

$$\frac{T_{R1} - T_1}{T_{R1} - T_0} = \Phi(\theta_1/f)$$

Let $\theta_1 = rc$.

Then $$\Phi(rc/f) = \frac{fb}{c} \ln \frac{b - 1}{b} \int_{c/f}^{0} \left(\frac{b - 1}{b}\right)^{f\lambda/c} \Phi(c/f - \lambda)\, d\lambda \quad . \quad . \quad . \quad (16)$$

In the limit $T_B \to \infty$, $b \to \infty$, and (16) becomes

$$\Phi(rc/f) = \frac{f}{c}\int_0^{c/f} \Phi(c/f - \lambda)\, d\lambda = \frac{f}{c}\int_0^{c/f} \Phi(x)\, dx = \frac{f}{c}\Psi(c/f) \quad . \quad . \quad (17)$$

The curves in Fig. 1 were calculated from equations (16) and (17) for $h/d = 0{\cdot}9$, and approximate corrections are given for other values of h/d.

When r satisfies equations (16) or (17), then after rc minutes at retort temperature T_{R1}, assuming instantaneous attainment of this temperature in the retort at time zero, the temperature at the centre of the can would be the same as that reached at the end of c minutes coming-up time of retort from T_0 to T_{R1}. When the F_C value of the coming-up time is negligible, rc is very nearly the equivalent value of the coming-up time in terms of time at T_{R1}, but when the F_C value is appreciable, rc is a low estimate of the value of the coming-up time. For it may be assumed, to a good approximation, that the actual course of temperature at the centre during the c minutes coming-up to T_{R1} is similar to the hypothetical course during rc minutes with the retort at T_{R1}, assuming no coming-up time, but the time scales are different in the ratio $c : rc$. Thus although the same temperature would be attained at the centre in each

case, the F_C value of the coming-up time would be more than that corresponding with the hypothetical rc minutes at T_{R1}, because the time units are f/r and f minutes respectively. If the F_C value of rc minutes at T_{R1} is x, the F_C value of the c minutes' coming-up is x/r, which is greater.

We require an amended value, r', of r to make the F_C values equal. This may be done by finding x as in Part I and then by finding a value r' such that $r'c$ minutes at T_{R1} gives $F_C = x/r$. In the procedure given above and illustrated in Example 3, it is necessary only to find u_H values, which are proportional to F_C values (see Part I). A conservative estimate of the value of the coming-up time is always obtained if this cumbersome correction is omitted.

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Chipping Campden, Glos.

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References

[1] Carslaw, H. S. & Jaeger, J. C., 'Conduction of Heat in Solids', 1947 (London: Oxford University Press)

[2] Gillespy, T. G., *J. Sci. Fd Agric.*, 1951, **2**, 107

[3] Bigelow, W. D., Bohart, G. S., Richardson, A. C. & Ball, C. O., *Bull. nat. Cann. Ass.*, 1920, 16L

[4] Patashnik, M., *Food Tech., Champaign*, 1953, **7**, 1

[5] Ball, C. O., *Univ. Calif. Publ. publ. Hlth*, 1928, **1**, No. 2, 15

[6] Riedel, L., *Mitt. Kältetech. Inst. Karlsruhe*, 1947, Nr. 1

[7] Schultz, O. T. & Olson, F. C. W., *Food Res.*, 1940, **5**, 399

THE NITROGENOUS EXTRACTIVES FROM FRESH FISH MUSCLE. II.*—Comparison of several Gadoid and Elasmobranch Species

By J. M. SHEWAN

The fractionation of the aqueous-alcoholic extracts of the muscle of several species of marine gadoids and elasmobranchs by displacement chromatography by means of ion-exchange resins, has shown the presence of 13 amino-acids in addition to creatine, creatinine, trimethylamine oxide and possibly glutathione in all species examined. Betaine and sarcosine appear to be confined to the elasmobranchs, and anserine and methylhistidine to the gadoids.

In a previous paper,[1] results were given of the fractionation of the nitrogenous bases in haddock muscle by means of ion-exchange resins. This technique has now been extended to over 25 species of both fresh-water and marine fish and the present paper records the results obtained for nine additional gadoids [cod (*Gadus morrhua*), whiting (*Gadus merlangus*), pollack (*Gadus pollachius*), coalfish (*Gadus virens*), poor cod (*Gadus minutus*), poutassou (*Gadus poutassou*), Norway pout (*Gadus esmarkii*), tusk (*Brosmius brosme*) and hake (*Merluccius vulgaris*)] and three elasmobranchs [skate (*Raja batis*), dogfish (*Acanthias vulgaris*) and smooth hound (*Mustelus vulgaris*)]. This classification is according to Jenkins, J. T., 'The Fisheries of the British Isles', 1942 (London: Warne).

Materials and methods

Most of the samples were caught off Aberdeen by the research vessel *Keelby*, and were extracted with alcohol, sometimes within an hour or two of catching, but more usually after overnight storage at 0°; or were frozen and cold-stored at − 30° for not more than 50 days until required. A few samples, namely poutassou, poor cod, Norway pout and tusk, were caught in the northern North Sea by the research vessel *Explorer* of the Marine Laboratory (Scottish Home Department), Aberdeen, were placed directly in alcohol after gutting, heading etc. within 30 minutes of capture, and subsequently treated on shore.

The methods used were essentially the same as those given in the earlier paper.[1] Absolute ethanol was added to the coarsely minced flesh to give a final concentration of 80% ethanol, the mixture was shaken up overnight, after which it was heated to 60° for 15 minutes and

* Part I: *J. Sci. Fd Agric.*, 1952, **3**, 394

filtered hot. Any crystalline deposit—usually a creatine–creatinine mixture—which appeared after cooling and standing at 0° overnight, was filtered off and the clear extract concentrated to half bulk.

In order to get rid of traces of protein, lipid, mucoids and inorganic salts, the crude extracts were given a preliminary enrichment and purification by passing them through a sulphonated cross-linked polystyrene resin which had been conditioned by washing, first with de-aerated 30% ethanol and then with de-aerated 60% ethanol. The last traces of lipid which remained on the column together with the amino-acids and bases were removed by washing the column with 60% ethanol until the effluent was free from fat, and the column was finally washed, first with de-aerated 60% ethanol and then with boiled distilled water. The bases and amino-acids were then displaced from this column with 0·070N-sodium hydroxide directly on to the first section of the multiple column used for the chromatographic fractionation. When the amino-acid front (which was observed as a paler-colour boundary on the resin) had reached almost to the bottom of the smallest column, fractions were collected, usually by an automatic fraction-collector, at the rate of 1 ml. per minute, in 3- or 10-ml. amounts.

Paper chromatography of the fractions and identification of the components

Samples of each fraction were spotted on Whatman No. 1 paper and one-dimensional chromatograms prepared, using three solvents: phenol–3% ammonia solution,[2] butanol–acetic acid[3] and phenol–hydrochloric acid.[4] The first of these solvents is particularly useful in separating the acid and neutral fractions, the second, the components that give the iodine reaction, valine and leucine, and the third the basic fractions.

The chromatograms were first treated with iodine by sublimation, the spots marked, the iodine was cleared with ammonia vapour and the paper then sprayed with the ninhydrin reagent to show up the amino-acids and other ninhydrin-reacting compounds.

In general, the displacement diagrams for all the gadoids examined were very similar, and so closely allied to those for the haddock that the behaviour of the components on the chromatograms, using three different solvents together with certain colour tests where possible, was considered sufficient proof of the identity of the compounds present. In a few instances, however, trimethylamine oxide and anserine were isolated and characterized more fully, the former by testing for trimethylamine by the *cis*-aconitic anhydride colour test[5] after reduction with titanium trichloride, the latter by showing chromatographically that β-alanine and methylhistidine were the only products present after hydrolysis.

Apart from the ninhydrin and iodine spot tests, additional colour tests were performed where possible, on duplicate paper-chromatograms or on aliquots of the fractions themselves. These included the Jaffé test for creatine and creatinine; the Paulay test for histidine, histamine and carnosine; and the Yudaev technique for differentiating carnosine, anserine, histidine and methylhistidine. Occasionally it was found more convenient to run two-dimensional chromatograms using phenol–ammonia solution and butanol–acetic acid in order to get good separations of histidine, methylhistidine, carnosine and anserine, and then apply the Paulay and Yudaev tests.

In both the haddock and in the gadoids described in this paper, a component, present usually in trace-to-weak amounts, appeared in the early fractions, but remained unidentified for some time. It was fortunate, however, that, in some of the species investigated, this component was present in moderate amount, and its identification was attempted. The portion containing this component was cut from several one-dimensional chromatograms, eluted with water and concentrated to small volume under vacuum. Chromatography of the concentrate gave only single spots with several solvents, showing the presence of only one ninhydrin-reacting compound which had R_F values almost identical to those of glutathione. After hydrolysis of an aliquot of the concentrated eluate, three spots were identified chromatographically as cysteic acid, glycine and glutamic acid. Additional proof of the identity of this component as glutathione was that it came off the column in a position approximating to its pK value,[6] namely 2·12.

Taking the gadoid species as a group, the following compounds were identified in the order of their displacement: glutathione, aspartic acid; threonine, serine, glutamic acid, proline; glycine, alanine, valine; leucine; creatine; β-alanine; trimethylamine oxide; creatinine; methylhistidine, anserine; ornithine, lysine, arginine; amines and ammonia. This sequence, which is similar to that of haddock,[1] accords fairly well with the pK values of the individual components (see Partridge[6]). As a typical example of the nine gadoids now being reported, the displacement diagram for whiting is given in Fig. 1. Three unidentified components, often occurring in fair quantity, were encountered in most of the gadoids examined.

These occurred between glycine and valine; between β-alanine and trimethylamine oxide; and between methylhistidine and anserine. These unidentified components are not shown in Fig. 1.

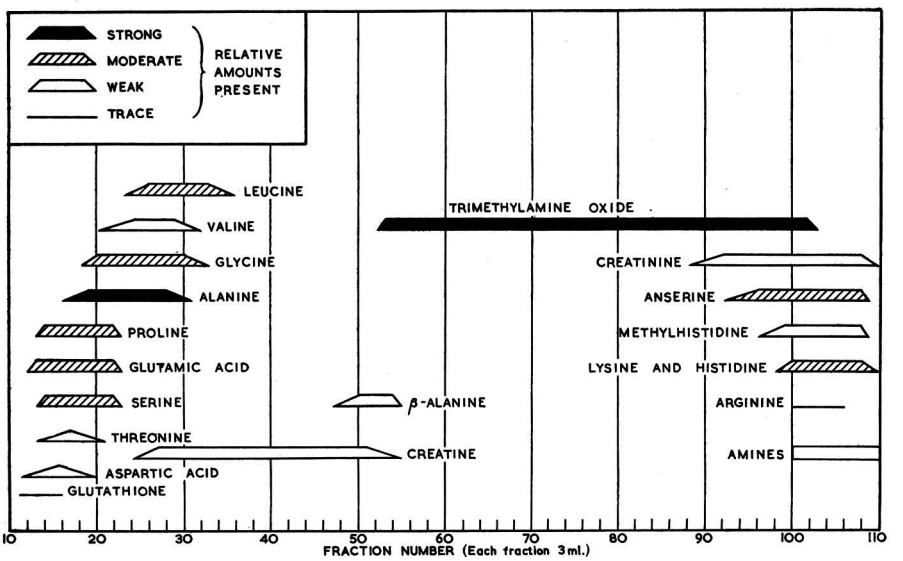

FIG. 1.—*Chromatographic fractionation of nitrogenous extract of whiting*

The three species of elasmobranchs all gave similar displacement diagrams, and only that for skate is given in Fig. 2. In order of displacement the compounds identified were: aspartic acid; betaine; threonine, serine, sarcosine, glutamic acid, proline; glycine, alanine, valine; leucine; creatine and β-alanine; trimethylamine oxide; creatinine, lysine, arginine, ornithine and amines.

Fractions 1–4 contained some betaine and traces of aspartic acid, and an unidentified component which was probably cysteine or glutathione. Fractions 5–25 each gave a single spot reacting only to Brante's[7] iodine reagent. From the chromatographic behaviour in various solvents, this component appeared to be betaine.

Betaine was isolated from fractions 5–25. These were bulked and evaporated down, and a white crystalline solid was obtained which, after further purification, had a m.p. (with decomposition) of 293°, and after mixture with pure betaine gave a m.p. of 293° (uncorr.) (Found: N, 11·79. Calc. for $C_3H_{11}O_2N$: 11·95%.)

Fractions 26–49 contained most of the acid and neutral amino-acids, glycine and β-alanine being present in large amounts, alanine in moderate amounts, and glutamic acid, valine and leucine in traces only. Of the three compounds giving the iodine reaction, creatine was present in traces, and sarcosine and betaine were present in moderate and large amounts respectively.

Fractions 50–70 contained large amounts of trimethylamine oxide only, which was isolated pure as previously described.[1] On being reduced with titanium trichloride it gave a positive *cis*-aconitic anhydride test for trimethylamine.[5]

Fractions 73–83 contained traces of arginine, lysine, creatinine and amines; moderate amounts of an unidentified ninhydrin-reacting compound; and large amounts of trimethylamine oxide.

It will be seen, therefore (Fig. 2), that the most striking feature of the composition of the extractives in the elasmobranchs is the predominance of betaine and trimethylamine oxide, and the presence of β-alanine, alanine, glycine and sarcosine in moderate amounts. Betaine, trimethylamine oxide, creatine and creatinine have already been recorded in certain elasmobranchs,[8–12] but the remaining compounds are now being recorded in these three species for the first time.

A comparison of the displacement diagrams for the gadoids and elasmobranchs shows

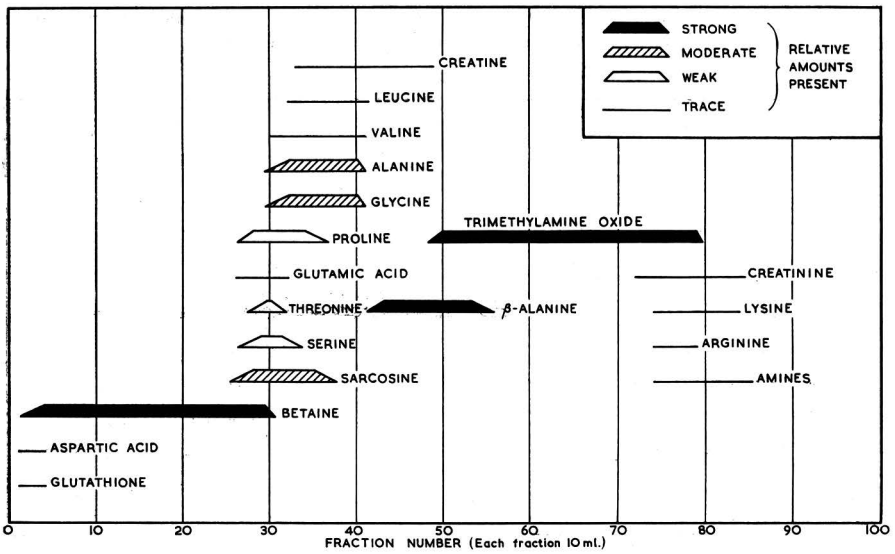

FIG. 2.—*Chromatographic fractionation of nitrogenous extract of skate*

many important differences. Qualitatively, betaine and sarcosine are present only in the elasmobranchs, and anserine and methylhistidine in the gadoids. Quantitatively, the gadoids show a greater predominance of the more basic components such as creatinine, methylhistidine, anserine, lysine and arginine, but in the elasmobranchs betaine and trimethylamine oxide are the two single components present in the greatest amounts.

Summary and conclusions

A qualitative analysis has been made of the basic fraction of the extractives in the fresh muscle of several species of marine gadoids and elasmobranchs. A striking feature of the results is the uniform pattern of composition in the species within each group, and the marked differences between the two groups as a whole.

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References

[1] Shewan, J. M., Fletcher, L. I., Partridge, S. M. & Brimley, R. S., *J. Sci. Fd Agric.*, 1952, **3**, 394
Consden, R., Gordon, A. H. & Martin, A. J. P., *Biochem. J.*, 1944, **38**, 224
[3] Partridge, S. M., *Biochem. J.*, 1948, **42**, 238
[4] Yudaev, H. A., *Trans. Acad. Sci. U.S.S.R.*, 1949, **67**, 1069
[5] Cromwell, B. T., *Biochem. J.*, 1950, **46**, 578; Palumbo, M., *Farmaco*, 1948, **3**, 675
[6] Partridge, S. M., *Analyst*, 1952, **77**, 955
[7] Brante, G., *Uppsala LäkFören, Förh.*, 1948, **53**, 301
[8] Suwa, A., *Pflüg. Arch. ges. Physiol.*, 1909, **128**, 421
[9] Kapeller-Adler, R. & Krael, J., *Biochem. Z.*, 1930, **221**, 437
[10] Berlin, E. & Kutscher, Fr., *Z. Biol.*, 1924, **81**, 87
[11] Spahr, W., *Z. Biol.*, 1936, **98**, 43
[12] Mohr, M. von, *Z. Biol.*, 1936, **98**, 276

THE CHEMICAL ESTIMATION OF VITAMIN-E ACTIVITY IN CEREAL PRODUCTS. I.—The Tocopherol Pattern of Wheat-Germ Oil

By P. W. RUSSELL EGGITT and L. D. WARD

Details are given of a method for determining the individual tocopherols and associated unidentified substances in oil extracted from cereal products. The technique depends ultimately on separating the tocopherols by paper chromatography by means of liquid paraffin B.P. and 75% aqueous ethanol as the stationary and mobile phases respectively. Typical results obtained with wheat-germ oil are quoted: they indicate that the tocopherol pattern of this oil consists of α-tocopherol, β-tocopherol and two unidentified substances, one of them possibly an unreported tocopherol.

The growing appreciation of the importance of vitamin E in the nutrition of livestock has led to intensified efforts to develop a reliable analytical method for estimating chemically the vitamin-E activity of both commercial and experimental rations. As the individual tocopherols differ widely in biological potency, it is essential to determine them separately and, with cereal products, clear differentiation between the tocopherols themselves and interfering substances is particularly important. The application, by Brown,[1] of paper chromatography to tocopherol assay has led to the development of an analytical procedure sufficiently straightforward for routine work.

After removing the lipids, sterols and carotenoids, Brown separated the tocopherols from vegetable oils by chromatography on strips of No. 1 Whatman filter paper impregnated with petroleum jelly. He reported that some destruction of the tocopherols occurred during his chromatogram runs and that, as a 16-hour development period was necessary for adequate separation of the γ- and δ-compounds, recovery figures of about 80% were the best obtainable. By using a solution of liquid paraffin B.P. in light petroleum to impregnate the paper strips, chromatography in an inert atmosphere and a simplified method of elution we have found that the separation can be made quantitative. The modified method also overcomes the difficulty caused by traces of an impurity, normally found in commercial grades of petroleum jelly, reacting with the Emmerie–Engel reagents. Full details of these modifications have already appeared.[2]

The complete analysis of a cereal product for vitamin-E activity involves the following steps:

(1) Solvent extraction of the oil.
(2) Removal of lipids by saponification.
(3) Removal of the bulk of the sterols by crystallization from methanol.
(4) Removal of carotenoids, sterols and other interfering substances by chromatography on columns of Floridin earth.
(5) Separation of the individual tocopherols and 'artifacts' by paper chromatography.
(6) Determination of the eluted tocopherols and 'artifacts' by the method of Emmerie & Engel.[3]

The methods available for each of these steps have been scrutinized and it is our aim to describe fully the practical details of the technique now standardized in this Laboratory and to illustrate the method with particular reference to the oil extracted from commercial wheat-germ meal. We have found that the tocopherol pattern of wheat-germ oil consists of α-tocopherol, β-tocopherol and two 'artifact spots', though previous publications recorded only one.[1, 4] These spots have been shown to be due to substances present in freshly milled wheat-germ and thus not to be true artifacts arising during the rather drastic purification procedure.

The methods of extracting tocopherols from natural materials are those normally used for fat extraction. Alcohol, light petroleum, ethyl ether, xylene and other solvents and solvent mixtures have been recommended, according to the nature of the product to be extracted. Vera[5] found light petroleum, benzene and trichloroethylene to be of equal value for extracting tocopherols from wheat germ, whether a Soxhlet apparatus or simple agitation was used. Purified ethyl ether has the advantage of rapidly extracting wheat germ at a relatively low temperature in a Soxhlet apparatus, and we have found that a commercial wheat-germ meal yields an oil of the same tocopherol content by this means as when it is extracted exhaustively by shaking repeatedly with fresh portions of ether in the cold. Ethyl ether used for this purpose must be peroxide-free and is conveniently purified, by the method of Werner,[6] immediately before use. The extraction is best conducted in the dark or in diffused light. *cyclo*Hexane has also proved a satisfactory solvent, but a prolonged extraction at its boiling point (17 hours)

produced an oil with a tocopherol content apparently 13% lower than that of the oil extracted from the same meal with ethyl ether.

Tosic & Moore[7] saponified vegetable oils in the presence of pyrogallol, to prevent losses of vitamin E by oxidation, and a slightly modified version of their method has proved to be the most reliable and convenient of all the alternatives investigated. Swick & Bauman[8] reported that excessive tocopherol recoveries were obtained with pyrogallol as the antioxidant and recommended the use of *p*-acetamidophenol, as suggested by Kjølhede.[9] Accordingly a series of analyses of wheat-germ oil were undertaken to compare the two antioxidants.

The two sets of results were in close agreement as to both the natural tocopherol content of the oil and the recovery of synthetic α-tocopherol supplements. It appears that the two antioxidants are equally effective and that excessive recovery values are not obtained with pyrogallol by the analytical procedure described below. In this Laboratory pyrogallol has been chosen as the antioxidant because it can be obtained in a high degree of purity. Its dark oxidation products also provide a useful indication that the ether extract of the saponification mixture has not yet been adequately washed; the last washing must be colourless.

Activated Floridin (a variety of fuller's earth) was used by Emmerie & Engel[10] for separating vitamin A and carotenoids from the tocopherols. Glavind, Kjølhede & Prange[11] boiled the earth, before use, with stannous chloride in hydrochloric acid to prevent tocopherol destruction during the chromatography. This procedure was also adopted by Brown,[1] the heated mixture being introduced into an adsorption tube and washed free of acid with ethanol (5 ml.) and benzene (5 × 5 ml.), after which the column was ready for the adsorption of carotenoids from a benzene solution. Swick & Bauman,[8] using such a column, found that the rate of flow during chromatography had an effect on the recovery of tocopherol.

We have studied methods of preparing and operating Floridin columns, using standard solutions of β-carotene and α-tocopherol, to make this stage in the analysis sufficiently reliable and reproducible for routine work. Floridin samples, even those marketed specifically for work with vitamin E, differ considerably in their chromatographic properties and in granularity, and it is advisable to test each sample before obtaining a sufficient quantity for use in a standard procedure.

Columns prepared according to the directions in the literature quoted were loaded with increasing quantities of β-carotene. The carotene appeared in the percolate when the deep-blue adsorption band produced by both carotene and vitamin A was restricted to the top few centimetres of the Floridin. The bottom half of the columns appeared to have little adsorbing power. This was due partly to the gradation in particle size during the settling of the suspension of earth poured into the tube, so that the finest particles occurred at the top, and partly to inadequate dehydration with ethanol. The performance of the columns was improved by increasing the number of ethanol washings before changing to benzene. Columns of the correct porosity at the benzene stage were inconveniently slow-running during the early stages of preparation, particularly before the removal of the hydrochloric acid, and the use of a carefully controlled pressure of nitrogen proved indispensable for routine work. The preparation of the adsorption columns was also improved on removing most of the concentrated hydrochloric acid by diluting the suspension with ethanol, centrifuging and resuspending the earth in ethanol before filling the tubes. Floridin columns prepared as described below will retain a much larger quantity of carotenoids than the relatively small quantity of xanthophylls contained in 1 g. of wheat-germ oil, and they are suitable for the analysis of feeding-stuffs containing considerable quantities of carotene. Smaller columns are not recommended for the wheat-germ-oil assay, however, as the Floridin earth is also required to remove some of the sterols.

Brown[1] removed most of the sterols, which form the bulk of the unsaponifiable matter of vegetable oils, by crystallization from methanol at — 10 to — 15°. Columns of Floridin earth, properly activated and dehydrated, were found to adsorb relatively large quantities of sterols from benzene solution. Up to 25 mg. of pure wheat-germ phytosterol was quantitatively adsorbed from benzene by the standardized technique, and the sterol was quantitatively recovered by elution with absolute ethanol. Hines & Mattill[12] found that columns of Florisil would adsorb cholesterol completely from pure solution, but not from extracts of animal tissues, owing to interference from vitamin A. Although our columns of Floridin XS were capable of removing both the carotenoids and the sterols from the non-saponifiable fraction of wheat-germ oil, we have retained Brown's procedure for removing most of the sterols before chromatography on the earth. The repeated crystallization of the sterols, though time-consuming, is simple to perform, results in no significant loss of tocopherol, and greatly increases the tolerance in the use of the Floridin columns, which is important with feeding-stuffs rich in carotenoids. The Floridin readily removes the phytosterol remaining after the crystallization.

Experimental procedure

Preparation of Floridin-earth chromatography columns

Reagents.—Floridin earth XS, 'Specially prepared for use in Emmerie's test for tocopherols', British Drug Houses, Ltd. (B.D.H.).
Stannous chloride, A.R., crystallized.
Hydrochloric acid, A.R.
Ethanol, absolute, redistilled from potassium hydroxide and potassium permanganate.
Benzene, A.R.

The adsorption tubes are of conventional construction, each with a body of 160 mm. × 9 mm., a reservoir of 60 mm. × 20 mm. and a capillary of 60 mm. × 0·5–1·0 mm. drawn into a jet (all diameters measured internally). The jet must not be drawn out so fine as to interfere with the final rate of flow of the column, which should be determined only by the porosity of the earth and the driving pressure.

Fig. 1 illustrates a stand designed to hold a dozen of these tubes so that a constant pressure of nitrogen can easily be applied to the columns. A pressure gauge or manometer is essential; the pressure is more easily controlled by a suitable gas-regulator on the nitrogen cylinder if a capillary leak to the atmosphere is provided in the pressure line. In addition, it is desirable to adjust the valve (or a screw-clip constriction) between the gas regulator on the cylinder and the apparatus to allow ample gas-flow to maintain the selected pressure with all the adsorption tubes in position, but to limit the maximum flow so that it is possible to remove an adsorption tube without the need to close its individual nitrogen-feed each time it is necessary to add more solvent. The loss of pressure on the line during these few moments prevents any other tube, in which the meniscus has reached the adsorbent, from 'sintering' while the attention is occupied

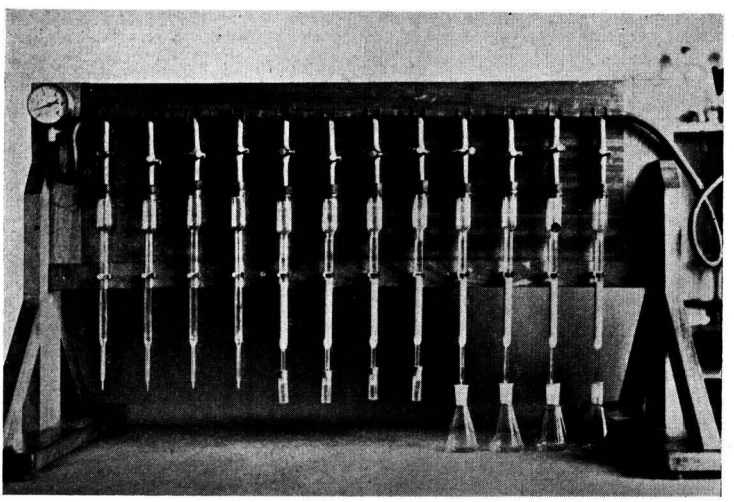

FIG. 1.—*Series of adsorption tubes designed for the application of constant pressure of nitrogen*

in replenishing the disconnected column. The tubes are conveniently supported by Terry clips (No. 80/100) and can be removed easily and snapped into position. Rubber pressure-tubing has proved satisfactory for connecting the T pieces forming the pressure line, and selected rubber bungs at the tops of the columns have been in use for many months without appreciable distortion. Care should be taken not to bring liquid benzene into contact with the bungs and to limit their exposure to the vapour to the periods when the columns are actually in use. Alternatively, the bungs may be replaced with special adaptors moulded entirely in polythene.

Sufficient adsorbent for a dozen tubes is prepared by boiling 30 g. of Floridin earth XS and 3 g. of stannous chloride with 100 ml. of concentrated hydrochloric acid. The mixture is maintained at the boil for 3 minutes and then cooled, and an equal volume of ethanol is added with

stirring before centrifuging. The supernatant liquid is poured off and the earth resuspended in absolute ethanol. This suspension, with continuous rapid stirring to ensure an even distribution of particle sizes, is used to fill the columns. The glass-wool pledgets in the chromatography tubes must first be covered with ethanol and freed from air bubbles by sucking ethanol up through the capillaries. The tubes should be filled until the final settled depth of the earth is about 8 cm. The columns are then washed with 5 × 5 ml. of absolute ethanol under a nitrogen pressure of 2 to 3 lb./sq. in. and are conveniently stored overnight in contact with the last portion of ethanol. After the pressure is released, the clips above each tube are closed when several cm. of ethanol still remain above the earth ; it is necessary to leave the jets of the capillaries dipping under ethanol to prevent air bubbles entering. This is done economically by attaching small (40 mm. × 10 mm.) specimen tubes (Fig. 1). Prepared columns may be kept in this condition for at least a week ; with longer storage any thin film of rubber from the bung adhering to the rim of the tube is readily wiped off with filter paper dipped in ethanol. The flow rate of an inconveniently slow column can usually be adjusted by stirring the top few millimetres of the finest earth into suspension and pouring it off. The columns are next washed with 5 × 5 ml. of A.R. benzene under a nitrogen pressure of 1 to 2 lb./sq. in. ; they are then ready for the chromatography. The rubber bungs should be replaced with polythene stoppers for any overnight storage after the benzene treatment. When the solutions for chromatography have been run on to the columns, they are developed with 3 × 5 ml. of benzene, with the pressure adjusted to between ½ and 1 lb./sq. in. so that the separation is complete in about 1 hour.

Extraction of the oil

The tocopherols are readily extracted from uncooked cereal products by the usual fat solvents. Ethyl ether in a Soxhlet extractor, used in diffused light, is particularly rapid with wheat-germ meal and is reliable if entirely free from peroxide. Ethyl ether (500 ml.) is conveniently purified, according to Werner,[6] by shaking it vigorously for 5 minutes in a litre bottle immediately after adding successively 4 g. of silver nitrate dissolved in 30 ml. of water and 2 g. of sodium hydroxide in 50 ml. of water, then pouring off the ether when the aqueous layer has settled. Light petroleum should also be purified before use.[12]

It has been shown that the volatile solvents can be removed without detectable loss of tocopherol if the surface of the solution is blown with a jet of nitrogen while the flask is being warmed on the water bath ; the flask need only be evacuated for a few minutes to assist in removing the last traces of solvent. This method has been found suitable for evaporating the solutions in ethyl ether, ethanol, methanol and benzene required during the analysis.

Saponification

Reagents.—Pyrogallol, A.R., 5·0% in absolute ethanol.
Potassium hydroxide, A.R., 40 g. in 27 ml. of distilled water.
Ethyl ether, A.R., purified with silver nitrate and caustic soda.

For each batch of wheat-germ oil to be assayed duplicate 1-g. portions are weighed into 150-ml. extraction flasks (B.24 ground-glass neck). To one sample is added 1·5 ml. of ethanol and to the other 1·5 ml. of a standard ethanolic solution of α-tocopherol (1 mg. per ml.). To each sample 2 ml. of 5% ethanolic pyrogallol is added, the flasks are warmed on a boiling-water bath and 0·5 ml. of the concentrated aqueous potassium hydroxide solution is run into each.

The mixture is kept at the boil for exactly three minutes with gentle shaking, a further 2 ml. of absolute ethanol is added and the flask cooled. The presence of ethanol at this stage is essential to prevent the formation of an emulsion during the extraction with ether. The flask is stoppered and shaken vigorously for a few minutes after the addition of 10 ml. of distilled water and then 25 ml. of purified ethyl ether. The mixed solutions are transferred to a 250-ml. separating funnel through a 5-cm. conical funnel and rinsed with purified ethyl ether from an all-glass wash-bottle. The aqueous layer is run off into the original 150-ml. flask for further extraction and the ether layer into a clean flask. The aqueous phase is extracted twice more, each time with 25 ml. of purified ether and 0·5 ml. of ethanol, added to break emulsions. The combined ether extract is washed four times with distilled water (20 ml.), cautiously during the first two washings and with vigorous shaking subsequently.

Removal of sterols

Reagent.—Methanol, redistilled.

The washed ether extract from each sample contains the unsaponifiable fraction of the original oil. It is evaporated to dryness by gently warming under a jet of nitrogen ; final drops of water are removed by evacuating the flask and immersing it in hot water for a few minutes.

It is then allowed to cool under the nitrogen jet. The residual solids are dissolved in 10 ml. of redistilled methanol by warming and the solution is transferred to a pre-cooled 50-ml. centrifuge tube provided with a pouring lip. The flask is rinsed with a further 2 ml. of methanol and the combined solution is cooled to − 12° and allowed to stand until crystallization is complete. The phytosterol crystals are centrifuged down in a pre-cooled centrifuge cup and the supernatant solution is poured off into a 50-ml. conical flask. The crystals in the centrifuge tube are redissolved in 5 ml. of methanol, which is first used to rinse out the original 150-ml. flask; the crystallization is repeated and the mother liquor is again removed. The process is repeated, each time with 5 ml. of methanol, until the sterols have been crystallized four times in all. A few sterol crystals pass over with the mother liquor at each stage, but the bulk are removed by this procedure, so that the sterols remaining are adsorbed subsequently by the Floridin earth without appreciably affecting the performance of the columns. (Under our conditions each stage in the above crystallization requires about 40 minutes.)

Chromatography on Floridin earth

The combined methanol solution from the sample is evaporated to dryness under the nitrogen jet in a 50-ml. conical flask and the last traces of solvent are removed by warming *in vacuo*. The flask is cooled to room temperature under nitrogen, 5 ml. of A.R. benzene is added from a pipette, the flask stoppered and the contents are gently swirled round the walls to ensure complete solution. Then 4 ml. of the bright-yellow solution is run on to a previously prepared column of activated Floridin earth. The column is developed with 3 × 5 ml. of benzene under a nitrogen pressure of ½ to 1 lb. per sq. in. The rate of flow is not very critical, but the complete separation should take not less than 45 minutes and the pressure should be adjusted so that the 15 ml. of benzene passes through the column in 45 to 75 minutes. The liquid that percolates is conveniently collected in a 50-ml. stoppered conical flask; it may be stored overnight in a refrigerator without loss of tocopherols.

Determination of total tocopherols

Reagents.—Ferric chloride (hydrated), A.R., 0·2% in absolute ethanol.
αα′-Dipyridyl, 0·5% in absolute ethanol.

The benzene solution is evaporated to dryness under nitrogen and the last traces of solvent are removed *in vacuo* as before. Absolute ethanol is pipetted into the cooled flask to give a solution of convenient concentration for determination of total tocopherols, and the flask is stoppered until required. The residue from 1 g. of fresh wheat-germ oil is dissolved in 40 ml. of ethanol and that from 1 g. of control-supplemented oil in the equivalent of 60 ml. (30 ml. diluted 2 : 1 before analysis). Several 1-ml. aliquots are taken from each ethanolic solution for the Emmerie & Engel determination. Each 1-ml. fraction is pipetted into a 50-ml. conical flask (B.14 ground-glass neck and stopper) and 2 ml. of absolute ethanol is added. In dim artificial light 0·5 ml. of ferric chloride solution and then 0·5 ml. of αα′-dipyridyl solution are blown in by means of bacteriological pipettes; the solutions are mixed and poured into 1-cm. spectrophotometer cells. The red colour is measured at a wavelength of 520 mμ two minutes after the dipyridyl pipette is blown out. The procedure is standardized against pure α-tocopherol. The ' recovery ' solution is then no longer required, but that from the unsupplemented oil is retained for determination of the tocopherol pattern by paper chromatography.

Quantitative paper chromatography

The individual tocopherols and artifact substances, making up the total tocopherols, are separated by chromatography on strips of No. 1 Whatman filter paper previously coated with liquid paraffin B.P. by dipping them in a 3% solution of the paraffin in light petroleum and allowing the solvent to dry. The chromatograms are developed in an atmosphere of nitrogen saturated with the vapour of 75% aqueous ethanol, which is used as the mobile phase. In a previous paper[2] we have described the preparation of the paper strips and the chromatography in experimental detail, including the location of the spots after a run, the elution, and the determination of the individual tocopherols and other substances reducing ferric chloride with the Emmerie–Engel reagents.

For wheat-germ oil the spotting solution is prepared by evaporating to dryness 30 ml. (in 3 × 10-ml. portions) of the ethanolic solution remaining after determination of total tocopherols; this is done in a 50-ml. centrifuge tube by heating on a water bath under the nitrogen jet. The tube is cooled under the jet and the tocopherol residue dissolved in 0·5 ml. of absolute

ethanol. With a micrometer syringe (Agla) 20-μl. portions of this solution, in 2 × 10-μl. aliquots, are spotted on to each of the prepared paper strips; the spots are allowed to dry between applications, keeping the strips horizontal and raised between the edges of Petri dishes where the spots are applied. Each strip is best spotted at a point that appears near the top of the vertical length of paper and just below the glass supporting-rod when the strip is in position for chromatography. The position will depend on the apparatus, but a sufficient length of paper is required between the origin and the solvent in the trough to obtain a reliable blank segment. We have found a distance of 4½ in. from the end of the paper suitable.

At least three papers are spotted for each sample and the chromatograms are developed side by side overnight (16–20 hours) in the dark. The central strip is then sprayed with the Emmerie–Engel reagents (dissolved in glacial acetic acid), allowed to hang in the dark for a few minutes, and used as a template for locating the tocopherol zones on the remaining quantitative strips. With oils of unknown tocopherol pattern a control chromatogram made with a mixture of α-, γ- and δ- tocopherols is required to identify the true tocopherol positions.

Segments 1½ in. long are often adequate for incorporating the tocopherol zones, especially from synthetic mixtures of tocopherols, but with vegetable oils the segments (including the blank) can be increased to 2 or 2½ in., according to the separation and spot size, without any other alteration in the technique of elution or of the Emmerie–Engel determination already described.[2]

The longer segments, when the separation permits, increase the tolerance in locating the tocopherol or artifact zones and allow for small variations in the movement of the solvent front.

In order to calculate the recovery from the chromatograms, the concentration of the spotting solution is determined. At the time the papers are spotted 20-μl. aliquots are pipetted from the micrometer syringe directly into each of four 50-ml. conical flasks; each is immediately washed down with 3 ml. of absolute ethanol and the total tocopherols are determined with the Emmerie–Engel reagents. The mean of the four results gives a reliable measure of the chromatogram loading independently of the accuracy with which the tocopherol residue was dissolved in 0·5 ml. of ethanol.

The necessity of conducting the chromatography in the absence of oxygen has already been emphasized, but really gas-tight chromatography chambers are not always readily available. A 24-in. length of 9-in.-diameter industrial glass pipe, flanged at either end, standing on a ground-glass plate and with a 9 to 2 in. flanged adaptor at the top, forms a satisfactory chamber. The end faces of the flanges are flat-ground to precision limits by the manufacturers; when lightly smeared with petroleum jelly their glass-to-glass joints are completely gas-tight. The 2-in. aperture of the adaptor is closed with a rubber bung carrying two glass tubes, one of them bending down the side of the chamber nearly to the bottom to facilitate rapid displacement of air with nitrogen. A small positive pressure usually builds up in the chamber during the run and it is advisable to bolt the adaptor lightly in position with a standard metal backing-flange.

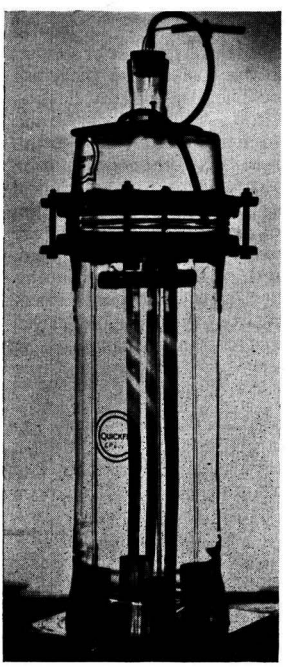

FIG. 2.—*Gas-tight chromatography chamber*

A robust scaffold to hold the polythene solvent trough and the chromatograms is easily made with glass and polythene rod, to avoid a fragile all-glass construction. The apparatus is illustrated in Fig. 2.

Results

After considerable experience in applying the method to wheat-germ oil we have found that in satisfactory assays the apparent recovery from the chromatograms always exceeds 95% and is usually very close to quantitative. In the typical sets of results that follow, the readings obtained with individual chromatograms are quoted in full to illustrate the satisfactory resolution of the total tocopherol mixture into its components. To calculate the percentage composition of the mixture, it is convenient to record the tocopherol content of the individual spots and of the spotting solution in terms of the optical density (O.D.) of the red colour produced with the Emmerie–Engel reagents. It was noted earlier[2] that, if the colour is developed

in a total volume of 4 ml. and with the Uvispek spectrophotometer at a wavelength of 520 mμ, a slit width of 0·1 mm., 1-cm. cells, and ethanol in the reference cell, the α-tocopherol equivalent is given by multiplying the optical density, less that of the blank, by 100·2. We prefer to redetermine this factor for each set of assays, by including quadruplicate aliquots from a standard solution of α-tocopherol as an overall check of the spectrophotometer and reagents, and to allow for any small changes due to variations in ambient temperature. The variations found are small and the mean of recent determinations gave a factor of 99·5. Hence the actual spot contents expressed as micrograms of α-tocopherol can be judged from the optical density figures in the Tables by merely moving the decimal point.

The 'top blank' segment, over which the solvent front has passed, is cut from the strip midway between the origin and the solvent in the trough: it normally gives a slightly lower figure than a blank segment taken below the limit of travel of the solvent front. This difference is much smaller with papers coated with liquid paraffin than with those coated with petroleum jelly.[2] By examining the variation in successive segments along blank strips of coated paper after developing with 75% ethanol, it has been found that the 'top blank' gives the better blank value for the positions taken up by the spots, and it has been used in the calculations recorded in the Tables.

Table I illustrates an analysis of the oil obtained from a freshly milled sample of commercial wheat-germ meal extracted for 2½ hours in a Soxhlet extractor with purified ethyl ether; a yield of 8·6% of oil was obtained under these conditions. The meal was extracted within 24 hours of milling.

Table I

Tocopherol pattern of oil extracted from freshly milled wheat-germ meal by Soxhlet extraction with purified ether

Moisture content of meal 12·0%
Yield of oil from meal 8·6% (2½ h.)

Total tocopherol content of 1 g. of oil A 2995 μg./g. } Mean 2990
" " " " " " " " B 2985 μg./g. }
Total tocopherol content of 1 g. of oil + 1·5 mg. of tocopherol

Recovery of added tocopherol 97%

Quantitative paper chromatography: optical density (O.D.) readings

Spotting solution, 20 μl.: 0·775, 0·772, 0·780, 0·769 } Mean 0·774

Reagent blank: 0·086, 0·085, 0·087, 0·086 } Mean 0·086

Total spotted on chromatograms (as O.D.) = 0·688

Segment	O.D. Paper 1	O.D. Paper 2	O.D. Paper 3	Mean O.D.	O.D. due to spot	Composition of tocopherol mixture, %
Top blank (run)	0·099	0·105	0·104	0·103	—	
Origin spot (unidentified)	0·201	0·205	0·196	0·201	0·098	14·2
α-Tocopherol	0·438	0·442	0·440	0·440	0·337	48·8
β-Tocopherol	0·294	0·298	0·298	0·297	0·194	28·0
Fast spot (unidentified)	0·173	0·161	0·160	0·165	0·062	9·0
Bottom blank (unrun)	0·104	0·107	0·111	0·107	—	
				Mean total eluted from paper	0·691	
				Total spotted	0·688	
				Recovery from chromatogram, %	100·5	

The results in Table II refer to an oil of lower potency obtained from another germ sample by prolonged extraction with *cyclo*hexane (17 hours) at the boiling point in a Soxhlet extractor of the loose-liner type. These are included to show the agreement between paper chromatograms loaded at different levels. The mean results from this Table can be compared with those published by Brown[1] for an oil with a closely similar total potency (Table III).

Table II

Comparison of the results from chromatograms loaded at different levels : wheat-germ oil obtained from commercial germ by prolonged extraction with cyclo*hexane*

Yield of oil (17-hour extraction with *cyclo*hexane) 9·5%
Total tocopherol content of oil 2600 μg./g.

Optical density (O.D) readings

Spotting solution, 20 μl.		Reagent blank	
0·666	Mean 0·660	0·067	Mean 0·067
0·658		0·068	
0·654		0·070	
0·660		0·064	

Total spotted on 20-μl. papers (as O.D.) = 0·660 − 0·067
= 0·593
Total spotted on 30-μl. papers (as O.D.) = 0·890

Segment	Paper spotted with 30 μl.			Paper spotted with 20 μl.		
	O.D.	O.D. less blank	Composition, %	O.D.	O.D. less blank	Composition, %
Top blank (run)	0·090	—		0·090	—	
Origin spot (unidentified)	0·190	0·100	11·2	0·160	0·070	11·8
α-Tocopherol	0·561	0·471	52·9	0·397	0·307	51·7
β-Tocopherol	0·324	0·234	26·3	0·255	0·165	27·8
Fast spot (unidentified)	0·175	0·085	9·6	0·142	0·052	8·8
Total eluted from paper		0·890			0·594	
Total spotted		0·890			0·593	
Recovery from chromatogram, %		100			100	

Table III

Comparison of the mean results from Table II with the figures published by Brown [1] *for an oil of similar total potency (percentages expressed to nearest whole number)*

	Present work : oil extracted with *cyclo*hexane	Brown's figures (1952)
Total tocopherol, μg./g.	2600	2620
Origin spot, %	12	not reported
α-Tocopherol, %	52	64
β-Tocopherol, %	27	27
Fast spot, % (Brown's artifact)	9	9

Table IV records the tocopherol pattern of an oil obtained by extracting a commercial germ-meal in the cold with ethyl ether.

The lower total potency of the oil obtained by extracting wheat-germ meal overnight with *cyclo*hexane in a Soxhlet extractor, compared with that obtained by a short extraction with ethyl ether, is not due to any significant destruction of tocopherols, or related substances, during the prolonged extraction, but mainly to dilution by extra fatty material extracted. The tocopherols are rapidly extracted and hence the shorter extractions are suitable for determining the total tocopherol content of a meal, though longer extraction is required to obtain more representative figures for wheat-germ oil.

Table V illustrates the total tocopherol contents of oils obtained by extracting 10-g. samples of commercial germ-meal with ethyl ether and with *cyclo*hexane in the dark for different periods of time. It will be noted that even with similar yields of oil the *cyclo*hexane gives slightly lower results for tocopherol contents than the purified ethyl ether.

Discussion

The origin spot

The origin spot has not previously been reported as forming part of the tocopherol pattern of wheat-germ oil, although we have found it accounts for between 11 and 14% of the total tocopherol figure obtained with freshly extracted oil. The spots are normally located by spraying the chromatogram with the Emmerie–Engel reagents and keeping it in the dark or in a dim

Table IV

The tocopherol pattern of wheat-germ oil extracted from commercial germ with purified ethyl ether in the cold

Total tocopherol content of oil 2970 μg./g.

Optical density (O.D.) readings

Spotting solution, 20 μl.		Reagent blank	
0·761	Mean 0·759	0·068	Mean 0·064
0·758		0·062	
0·759		0·063	
0·759		0·063	

Total spotted on chromatograms (as O.D.) = 0·695

Segment	O.D. Paper 1	O.D. Paper 2	O.D. Paper 3	Mean O.D.	O.D. less blank	Composition, %
Top blank (run)	0·086	0·079	0·081	0·082	—	—
Origin spot	0·173	0·173	0·170	0·172	0·090	12·9
α-Tocopherol	0·436	0·439	0·442	0·439	0·357	51·4
β-Tocopherol	0·285	0·283	0·276	0·281	0·199	28·6
Fast spot	0·132	0·130	0·132	0·131	0·049	7·1
		Mean total eluted from paper			0·695	
		Total spotted			0·695	
		Recovery from chromatogram, %			100	

Table V

Comparison of solvents used for the Soxhlet extraction of commercial wheat-germ meal

Duration of Soxhlet extraction, h.	Oil extracted from meal, %	Total tocopherol content of oil, μg./g.	Total tocopherol content of meal, μg./g.
*cyclo*Hexane			
2½	8·75	2780	243
4	9·16	2660	244
8	9·20	2730	251
17	9·53	2600	247
Purified ethyl ether			
2½	8·90	2970	264
4	9·06	2860	259
6	9·21	2780	256
8	9·33	2820	263

light for a few minutes until the spots are distinct. The origin spot develops much more slowly than the three mobile spots, even when the test is made more sensitive by dissolving the spraying reagents in glacial acetic acid instead of in ethanol.[2] This difference possibly explains how the material remaining on the origin came to be overlooked in the published analyses[1] and the consequent low recoveries. We have found that, under our conditions, losses of tocopherols of up to 12% also arise, during a 16-hour run, if the air in the chromatography chamber is not displaced with nitrogen. It appears possible that these two factors together account for the losses of 23% on wheat-germ-oil chromatograms reported by Brown.[1]

Although the origin spot is slow in appearing on the sprayed chromatograms, the intensity of the colour produced by the solution of material eluted from the spot increases very little after the two-minutes' reaction time allowed for the standard Emmerie–Engel test.

The observation by Brown[1] that old ethanolic solutions of pure tocopherols produce a faint-pink spot on the origin when chromatographed and sprayed with the Emmerie–Engel reagents has been confirmed. This suggested that the wheat-germ origin spot might be due to an artifact produced either during the storage of either the oil or the germ meal itself, or during the extraction and rather drastic analytical procedure. Table IV, however, records that from a sample of wheat-germ meal gently extracted with ethyl ether in the cold, within 24 hours of milling, the origin spot contributed 14% to the ferric chloride-reducing material remaining after the Floridin earth chromatography. Artifact formation by modification of a tocopherol

during saponification or chromatography is considered unlikely, as no significant difference was found between chromatograms prepared from a sample of pure α-tocopherol submitted to the complete analytical treatment and control chromatograms.

The origin spot, on a wheat-germ-oil chromatogram, gives an instantaneous pink colour when sprayed first with sodium carbonate solution and then with the diazotized *o*-dianisidine reagent of Weisler *et al.*;[13] it is the only spot in the wheat-germ tocopherol pattern to give any reaction with the reagent. This confirms previous results that γ-tocopherol and δ-tocopherol are both absent from wheat-germ oil.[1, 15]

We consider the spot, which neither moves off the start line nor spreads beyond the area originally occupied by the spotting solution applied to the paper, to be produced by an unidentified non-tocopherol substance, or a mixture of such substances, normally present in fresh wheat-germ oil. Similarly, the fast spot is produced by an unidentified normal constituent of the oil, and neither can properly be called an artifact. No attempt has been made to ascertain the identity of the material forming the origin spot.

The fast spot

Brown[1] reported a fast-moving artifact spot on his chromatograms of the wheat-germ-oil tocopherol mixture, contributing 9% of the total tocopherols. It did not react with diazotized *o*-dianisidine and is clearly formed by the same substance as the fast spot on our chromatograms. Using papers coated with petroleum jelly, Brown found his artifact had an R_F value 0·09 greater than that of δ-tocopherol, whereas under our conditions the fast-spot R_F is greater by only 0·03. The different mobility is not sufficient to resolve the two substances on our chromatograms and a single spot is obtained in the δ-position when the wheat-germ-oil mixture is supplemented with δ-tocopherol before chromatography. Thus the fast spot behaves similarly to δ-tocopherol on the chromatograms, but does not couple with diazotized *o*-dianisidine.

With the four known naturally occurring tocopherols, increasing methylation of the benzene ring in the chromane nucleus appears to reduce the R_F value step-wise with each additional methyl group, the position of substitution having little effect. Typical R_F values under our conditions are: α, 0·24; β, 0·48; γ, 0·48; δ, 0·65. Thus both β- and γ-tocopherol, the dimethyl isomers, run to the same position on the chromatogram. This suggests that an isomer of δ-tocopherol would run close to the δ-position.

As both γ- and δ-tocopherols couple with diazotized *o*-dianisidine whereas α- and β-tocopherols do not, it appears that the absence of substitution at position 5 is required to enable the coupling to take place. The fast spot does not couple; hence if it proves to be due to a tocopherol the unidentified substance would seem likely to be 5-methyltocol.

The presence of an ε-isomer might explain the reported presence of 5% of δ-tocopherol in the wheat-germ tocopherol mixture,[14] although paper chromatography and the sensitive dianisidine test now show it to be absent. On the other hand, Quaife[15] could find only one non-α-tocopherol in wheat-germ oil by chromatography of the nitroso-derivatives on zinc carbonate–Celite, although her technique would not have detected an esterified tocopherol.

Cronheim[16] found that the positions of the absorption maxima and minima of the spectra of nitrosophenols follow two definite rules: (1) the wavelength of minimum absorption increases with the number of substituents in the benzene ring and (2) the wavelength of maximum absorption depends upon the position relative to the hydroxyl group of the nearest substituent, except the nitroso group itself. Quaife[15] showed that the nitrosotocopherols appear to obey these rules, and from the figures given in her paper one would expect 5-methyltocol to form a nitroso-compound with a spectrum combining the absorption minimum of nitroso-δ-tocopherol (340 mμ) with the absorption maximum of either nitroso-β- or nitroso-γ-tocopherol (410–415 mμ).

Preliminary experiments have shown that the fast-spot substance does form a yellow nitroso-derivative, which has an absorption maximum at 415 mμ, although at present the nitroso-compound has not been obtained in sufficient concentration to fix accurately the position of the absorption minimum, as this occurs in a region (340–350 mμ) where the spectrophotometer is relatively insensitive.

Fig. 3 illustrates the absorption curve, between 380 and 500 mμ, obtained with the nitroso-compound from approximately 65 μg. of the fast-spot substance (expressed as the Emmerie–Engel equivalent of α-tocopherol), and dissolved in 7·5 ml. of light petroleum (60–80°) to provide just sufficient solution to fill a 2-cm. spectrophotometer cell.

This material was obtained by chromatographing larger quantities of the wheat-germ tocopherol mixture (after the usual purification) on sheets of filter paper coated with liquid paraffin B.P. Sheets 5 in. wide were used and the spotting solution was applied as a band near the top of the paper. The band of fast-spot material was located on each chromatogram, after developing

with 75% ethanol, by cutting off a narrow strip ($\frac{1}{2}$ in.) from each side of the sheet and spraying with the Emmerie–Engel reagents dissolved in glacial acetic acid. The fast-spot zones were eluted with absolute ethanol. A difficulty arose on concentrating the eluates to a small volume, owing to the separation of a liquid-paraffin phase, but sufficiently concentrated solutions were obtained to prepare the nitroso-derivative according to the method of Quaife.[15] A blank was prepared for the spectrophotometer reference cell by treating in the same manner the extract from an equal area of coated filter paper from the chromatograms below the limit of travel of the solvent front. The blank was colourless, whereas the fast-spot nitroso-derivative, extracted from the nitrous acid mixture with 7·5 ml. of light petroleum, gave a yellow solution.

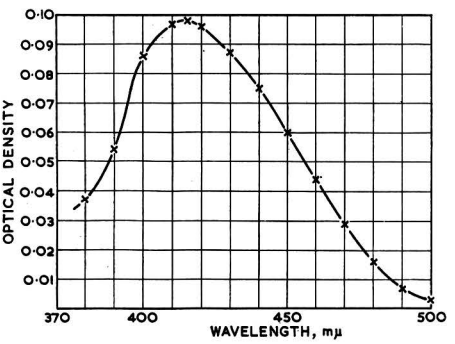

FIG. 3.—*Absorption spectrum of the 'fast-spot' nitroso-compound*

An Emmerie–Engel determination with an aliquot of the final concentrated eluate revealed that about 65 μg. of the fast-spot material was available for the nitroso reaction, and the depth of colour obtained, expressed as optical density, agreed well with that calculated for a similar quantity of γ-tocopherol after checking the procedure with the latter.

From the behaviour of the fast-spot substance on paper chromatograms, its failure to couple with diazotized *o*-dianisidine, and the absorption spectrum of its nitroso-compound formed with nitrous acid, it appears likely that it will prove to be 5-methyltocol. It is hoped to publish later the results of a more detailed investigation into the nature of this substance.

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References

[1] Brown, F., *Biochem. J.*, 1952, **51**, 237; 1952, **52**, 523
[2] Russell Eggitt, P. W. & Ward, L. D., *J. Sci. Fd Agric.*, 1953, **4**, 176
[3] Emmerie, A. & Engel, C., *Rec. Trav. chim. Pays-Bas*, 1938, **57**, 1351
[4] Brown, F., *J. Sci. Fd Agric.*, 1953, **4**, 162
[5] Vera, O. R., *Rev. Fac. Cienc. quím. La Plata*, 1947, **22**, 231
[6] Werner, E. A., *Analyst*, 1933, **58**, 335
[7] Tosic, J. & Moore, T., *Biochem. J.*, 1945, **39**, 498
[8] Swick, R. W. & Baumann, C. A., *Analyt. Chem.*, 1952, **24**, 758
[9] Kjølhede, K. T., quoted by Wanntorp, H. & Nordlund, G., *Ark. Kemi. Min. Geol.*, 1947, **25**, 1
[10] Emmerie, A. & Engel, C., *Rec. Trav. chim. Pays-Bas*, 1939, **58**, 283
[11] Glavind, J., Kjølhede, K. T. & Prange, I., *Kem. Maanedsblad*, 1942, **23**, 43
[12] Hines, L. R. & Mattill, H. A., *J. biol. Chem.*, 1943, **149**, 549
[13] Weisler, L., Robeson, C. D. & Baxter, J. G., *Analyt. Chem.*, 1947, **19**, 906
[14] Stern, M. H., Robeson, C. D., Weisler, L. & Baxter, J. G., *J. Amer. chem. Soc.*, 1947, **69**, 869
[15] Quaife, M. L., *J. biol. Chem.*, 1948, **175**, 605
[16] Cronheim, G., *J. org. Chem.*, 1947, **12**, 20

THE BLEACHING OF CAROTENE BY A LIPOXIDASE–LINOLEATE SYSTEM

By J. A. BLAIN, J. HAWTHORN and J. P. TODD

A system for studying the coupled oxidation of carotene by lipoxidase and similar fatty acid oxidases is described. The experimental characteristics of the system are illustrated, and particular attention is paid to the effect of pH on the observed reaction velocities. The system presents certain advantages over previous systems of a similar nature.

Introduction

A recent review by Holman & Bergstrom[1] surveys the fairly extensive literature of lipoxidase and other unsaturated-fat oxidase systems. The technical importance of the lipoxidase-coupled bleaching of carotenoids has been known since 1934, when Haas & Bohn[2] patented their process for the bleaching of the yellow pigments in bread doughs by the use of soya preparations. The reactions of these unsaturated-fat oxidases have been shown to be responsible for such diverse and undesirable effects as losses of vitamin A in dried lucerne[3] and in butter,[4] the development of rancidity in herring muscle,[5] and peroxide formation on low-temperature storage of hog adipose tissue.[6, 7] Additional technical interest is given to carotenoid bleaching by such recent publications as those of Irvine & Winkler[8, 9] on colour losses in macaroni doughs, and that of Rank & Hay[10] on a bread-baking process with unbleached untreated flour.

The only unsaturated-fat oxidase so far characterized and isolated in a pure form is soya-bean lipoxidase,[11] although it is known that haem proteins are associated with similar reactions in animal tissues.[5, 7]

In early studies of lipoxidase activity,[12–15] the bleaching of carotenoids was used for assay purposes. These methods have been largely superseded by the spectrophotometric assay of Theorell, Bergstrom & Åkeson,[16] which utilizes the absorption band at 234 mμ to measure the degree of diene conjugation produced by the enzyme in an unsaturated fatty acid substrate. This method is not subject to certain errors of the carotenoid bleaching methods which are associated with variations in the degree of dispersion of the substrate and the carotenoid. Recently, Irvine & Anderson[17] proposed a manometric technique for the estimation of lipoxidase activity in wheat.

Nevertheless, the assessment of technical processes involving the coupled enzymic bleaching of carotenoids, and the study of their reaction conditions and mechanisms, cannot be carried out solely by the spectrophotometric method. In previous carotenoid assay systems little systematic attention was given to some of the factors. The work described in the present paper was carried out in an attempt to establish valid conditions for the quantitative study of these bleaching processes.

The unusual feature of the lipoxidase reaction is that it takes place in two phases, the coupled reactant and the fatty substrate being insoluble in the aqueous enzyme phase. The observed reaction velocities will therefore reflect the degree of dispersion of the reactants, and consequently any method must seek to standardize the reactant dispersion.

The enzyme is known to have three substrates, namely linoleic, linolenic and arachidonic acids, which are water-soluble only at high pH values. The spectrophotometric method overcomes the dispersion difficulty by the use of sodium soaps as substrate at pH 9, where an apparent activity optimum is obtained and where dispersion is reproducible. In the carotene-bleaching method, stabilizers of various kinds,[13] as well as surface-active agents, have been used, but these may introduce activating or inhibiting effects which vary with concentration and pH.[18] The method to be described avoids the use of such agents.

In previous measurements of carotenoid bleaching, and particularly at acid pH values, observations were made on solutions which were turbid to a greater or less degree unless a stabilizer was used. Alternatively, some workers extracted the pigment with a suitable solvent after carrying out the reaction, but the procedure was tedious. In the proposed system extraction is avoided and measurements are made on clear solutions. As a result of this, a method of activity measurement of crude soya extracts is proposed which may be used in the study of technical processes involving carotene bleaching by lipoxidase or by similar systems.

Reagents and apparatus

The following reagents and apparatus are required:

Extraction buffer, pH 4·5.—114 ml. of 0·1N-acetic acid and 86 ml. of 0·1N-sodium acetate.

Reaction buffer, pH 6·0.—12 ml. of M/15-disodium hydrogen phosphate and 88 ml. of M/15-potassium dihydrogen phosphate, diluted to 500 ml. with distilled water.

Alternative reaction buffers, pH 3·0 *to* 8·0.—Where observations over a fairly wide range of pH are required, McIlvaine's[19] citrate/phosphate buffer system has been found convenient. Five volumes of McIlvaine's mixtures of 0·2M-disodium hydrogen phosphate and 0·1M-citric acid are diluted to fifty volumes. The pH is determined in the diluted buffer in the presence of the appropriate quantities of substrate and carotene solutions. Buffer strengths generally should be about M/75, since higher concentrations tend to cause precipitation of carotene.

Carotene solution.—1·5 mg. of carotene (98% β-carotene from The British Chlorophyll Co. Ltd.) from a freshly opened ampoule is dissolved in 100 ml. of a mixture of acetone/ethanol (75/25 v/v).

Aqueous caustic soda.—A 20% solution in distilled water is used to stop the enzyme reaction.

Alcoholic caustic soda.—4 g. of caustic soda pellets are dissolved in 5 ml. of warm distilled water and diluted to 100 ml. with ethanol. The solution is standardized in the usual way.

Sodium linoleate substrate.—Ethyl linoleate is prepared from cottonseed oil[20] and stored in evacuated ampoules at − 20°, each ampoule containing 0·44 g. of ethyl linoleate, which is equivalent to 0·40 g. of linoleic acid. A slight excess of the alcoholic N-caustic soda solution is added to an opened ampoule and mixed. The solution is allowed to saponify overnight and diluted to 400 ml. with distilled water. One ml. of this solution contains 1 mg. of linoleic acid. The substrate thus prepared must be kept at 0° to 4° when not in use, and should be used within 14 days of preparation.

Colour measurements.—The Hilger Spekker absorptiometer, with Ilford spectrum violet filters (No. 601) and 40-mm. cells, is used.

Reaction flasks.—The reaction is carried out in 200-ml. conical flasks, which are set aside for this purpose after being soaked overnight in a strong solution of caustic soda. They are thoroughly rinsed with distilled water before drying for use. After use, rinsing and drying are repeated before re-use. Soaps and detergents are to be avoided for cleaning glassware, since the presence of traces of such surface-active agents has been found to give rise to inaccuracies. After repeated use there is a slight tendency for carotene to be precipitated on the surfaces of the flasks. This is removed from time to time by rinsing with acetone before preparing the flasks in the usual way.

Flask shaker.—In carrying out the additions to the reaction buffer, it has been found that reproducibility is enhanced by a simple mechanical-shaking device as shown in Fig. 1. This consists of a circular horizontal plate, of the same diameter as the base of the flask, fitted with a raised edge and mounted 5 mm. off centre to the vertical driving shaft. The flask is located on the plate by means of an annulus of rubber tubing stiffened by passing a length of thick wire solder through it. It is held in position by means of two wires soldered to the plate and diametrically opposite one another. A small motor drives the plate at about 300 r.p.m. When additions are made, the pipette is introduced under the surface of the liquid and the violent agitation produced gives very rapid mixing of the reagents and uniformity of dispersion.

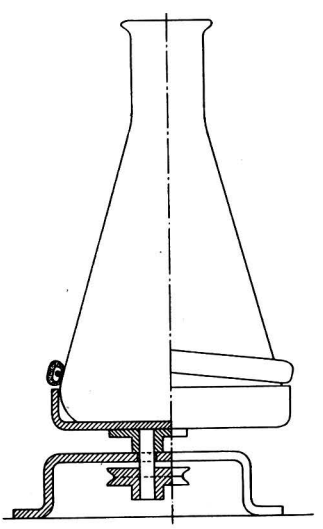

FIG. 1.—*Flask-shaking device*

Method

The extraction of lipoxidase from soya flour.—The coarsely ground material is defatted for six hours in a Soxhlet apparatus with *n*-pentane or a light petroleum fraction boiling below 40°. The defatted material is ground to pass a 60-mesh sieve, and 5 g. suspended in 50 ml. of extraction buffer. The mixture is shaken for one hour, then centrifuged for ten minutes at 2000 r.p.m. and decanted through a No. 1 Whatman filter paper. The solution thus prepared loses little activity during the course of a few weeks when stored at 0–5°.

Assay.—The reaction is carried out in the prepared conical flasks, the additions being made in the following order:

Addition	Reaction flask	Blank flask
1	Reaction buffer, 50 ml.	Reaction buffer, 50 ml.
2	Substrate solution, 1 ml.	Aqueous sodium hydroxide, 2 ml.
3	Carotene solution, 2 ml.	Substrate solution, 1 ml.
4	Enzyme solution, x ml.	Carotene solution, 2 ml.
5	Aqueous sodium hydroxide, 2 ml.	Enzyme solution, x ml.

The reaction buffer is added to the flasks and left overnight to equilibrate with atmospheric oxygen before use. During the reaction it should be at a temperature of 20° (± 2°). The volume of enzyme solution added should be about 0·05 to 0·30 ml., and a microburette to deliver under the surface of the liquid is necessary. In carrying out the reaction there should be no delay between additions after the substrate has been added. As soon as the enzyme has been added a stop-watch is started and after exactly one minute caustic soda is added to stop the reaction. The percentage of carotene destroyed is then read in the absorptiometer with 40-mm. cells. Gas bubbles in the solutions should be allowed to disperse before readings are taken. In bright daylight some slight bleaching of the alkaline solution takes place, and it is therefore desirable to make the observations within an hour of carrying out the treatment. The solution is cloudy while the reaction is proceeding at acid pH values, but becomes clear on the addition of alkali.

Readings are obtained for at least three levels of enzyme concentration, so that the curve joining them (plotted against carotene destruction) passes through the 50% destruction level. From the curve, the volume of enzyme solution required to destroy 50% of the carotene present (i.e. 0·015 mg. of carotene) is read. This volume represents one unit of activity for the system described.

Experimental

The main characteristics of the proposed system are illustrated in Figs. 2 to 10.

Optimum pH.—The optimum pH of lipoxidase has been the subject of controversy. Holman,[21] using crystalline lipoxidase and a sodium linoleate substrate with the spectrophotometric assay, found the optimum to be pH 9, and Smith & Sumner,[22] using an emulsified ester substrate, found an optimum at pH 6·5 and suggested that Holman's figure represented increasing availability of substrate with pH for his system, but direct experimental evidence for this has not been given. Holman[1] doubts the possibility of measuring the inherent optimum of lipoxidase until a substrate, water-soluble over the whole pH range of interest, can be obtained. More recently, Fukuba[23] has studied the substrate properties of polyoxyethylene linoleate, whose interfacial tension against xylene was found to be independent of pH, and observed an optimum between pH 6·5 and 7·0.

In the system described here, the omission of carotene and the measurement of diene conjugation by the band at 234 mμ gave a curve that was essentially similar to that of Holman, activity increasing with pH towards an optimum at about pH 9, although the overall substrate concentration (1 mg. of sodium linoleate in 53 ml. of reaction mixture) was about one-eightieth of that of Holman (2 mg. in 1·2 ml. of reaction mixture). When carotene was introduced into this system the curve shown in Fig. 2 was obtained. Similar curves were observed whether the treatment was carried out in citrate/phosphate or phosphate buffers.

On this evidence it appeared that the carotene, and the acetone/ethanol solvent introduced with it, must alter the characteristics of the system. A study of the carotene solution in the quantities used added to the phosphate/citrate buffer showed that the absorption, and hence presumably the dispersion of the carotene, did not change significantly with pH. The data plotted in the upper part of Fig. 3 show the results obtained, and it may be noted at this stage that carotene dispersion in buffer in the absence of substrate tends to be unstable. Strict attention to technique is necessary to obtain these observations. Fig. 4 shows a spectrophotometric comparison, on an $E^{1\%}_{1cm.}$ basis, of the absorption of a specimen of carotene in a chloroform solution and in the aqueous reaction system at pH 8, appropriate corrections having been made for the absorption of the acetone. Clearly, the carotene in the aqueous solution is in a different state of dispersion from that of carotene in organic solvents.

At acid pH values the solution of substrate in the reaction buffer becomes turbid almost immediately owing to precipitation of linoleate. With the appropriate buffer blanks, measurements of Spekker absorptiometer readings showed that the turbidity increased very rapidly

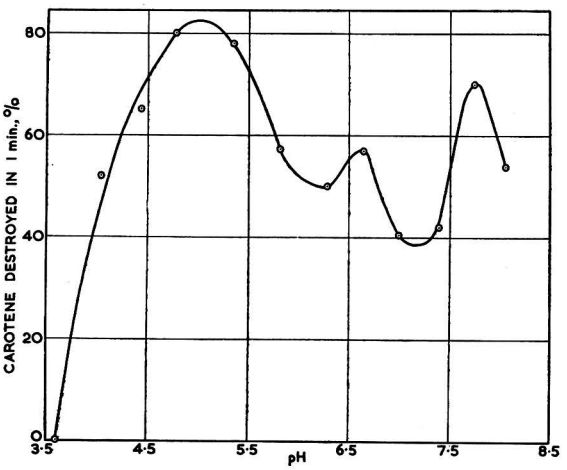

FIG. 2.—*Activity curve of the system*

during the first minute after the addition of the substrate and afterwards continued to increase gradually. The lower curve in Fig. 3 shows this development of turbidity with pH in the buffer, in the absence of enzyme and carotene, two minutes after the addition of substrate. It would appear that precipitation of linoleate with decreasing pH value begins at about pH 7·7, but the general form of the curve, although indicative of unexpected complexities, offers no explanation of the peaks on the pH–activity curve.

When, as shown in Fig. 5, the absorption of the complete system in the absence of enzyme is studied, a curve is obtained with maxima and minima related inversely to those of the pH–activity curve. As Fig. 3 shows, the carotene readings do not change appreciably with pH,

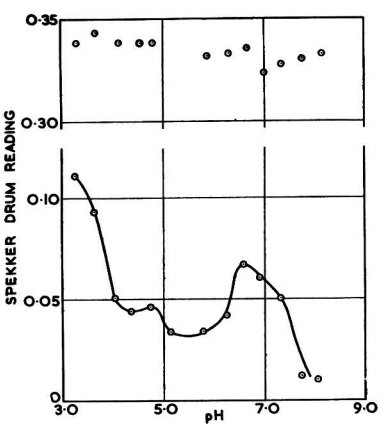

FIG. 3.—*Absorption characteristics of the components of the system*
Upper plot: Variation in optical density of carotene in phosphate/citrate buffer with pH
Lower curve: Variation in substrate turbidity in phosphate/citrate buffer with pH

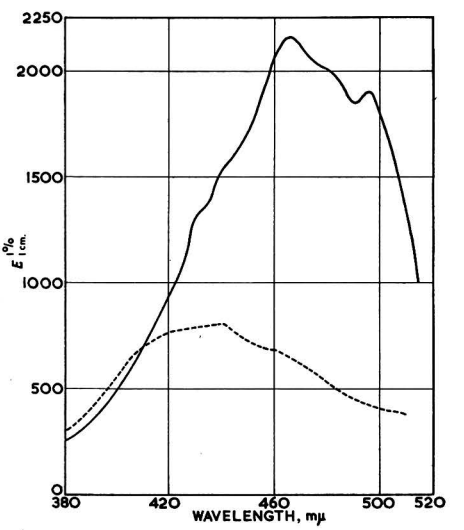

FIG. 4.—*Absorption curves of carotene*
Upper curve: In chloroform solution
Lower curve: In phosphate buffer dispersion, pH 8

but the substrate-turbidity readings change markedly. It would therefore be expected that the absorption of the complete system (i.e. buffer with substrate and carotene in absence of enzyme) will parallel the substrate-turbidity curve. This, however, does not take place, and it follows that the presence of substrate modifies the state of dispersion of the carotene. Moreover, the inverse relationship of the curves shown in Figs. 2 and 5 indicates that this modification is related to the observed activity of the system. It appears, therefore, that the pH effects observed reflect the mutual availability of enzyme, substrate and carotene. It will be noted that the maxima and minima in Fig. 5 show a slight pH shift with respect to the minima and maxima in Fig. 2, but this would be expected, since the inherent activity of the enzyme will vary over the pH range. The precise position of any single peak on the pH–activity curve will reflect availability of substrate and carotene, modified by inherent activity of enzyme.

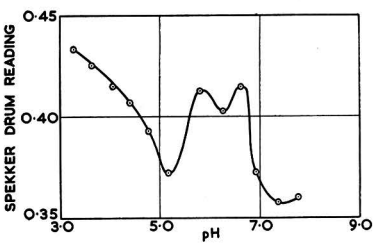

FIG. 5.—*Variation in optical density of the complete system (enzyme absent) with pH*

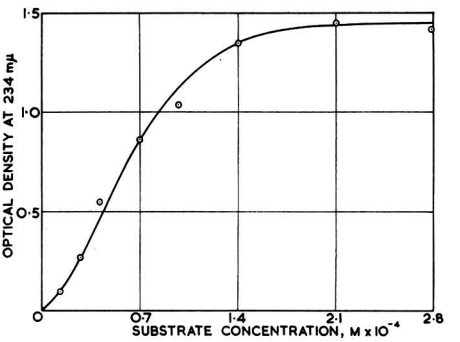

FIG. 6.—*Substrate concentration/velocity curve for the reaction system in absence of carotene at pH 8*

Effect of substrate concentration.—Fig. 6 shows the diene conjugation (as measured spectrophotometrically by the peak at 234 mμ) produced by the interaction for one minute of a constant quantity of enzyme with increasing quantities of substrate, in the absence of carotene, in the proposed reaction system at pH 8. The form of the curve is as would be expected—the activity increases to a maximum and afterwards remains constant.

Fig. 7 shows the destruction of carotene under the same conditions of substrate concentration, enzyme concentration, pH and time as those in Fig. 6. A maximum is reached at a substrate concentration of approximately 0·01M $\times$ 10^{-2}, but the carotene destruction at this substrate concentration is only very slightly greater than at 0·007M $\times$ 10^{-2}, which is the proposed level for the system. In the region of this level, variations in substrate concentration of up to 20% have only a minor effect on the carotene destruction. The existence of an apparent substrate optimum in carotene-bleaching systems has been noted by previous workers, and was related by Balls, Axelrod & Kies[15] to the presence of an activator in crude soya extracts. A study by the present workers of the substrate relationships of lipoxidase-active wheat-germ extracts did not show such an optimum in the presence of carotene.

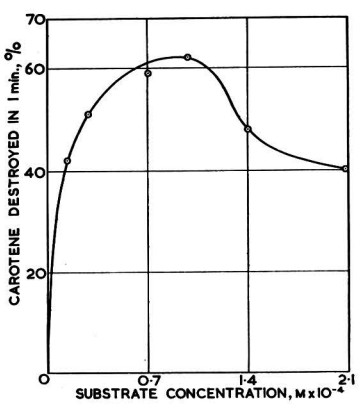

FIG. 7.—*Substrate concentration/carotene destruction curve for the system at pH 8 with a one-minute reaction time*

Holman[1] gives a figure of 160 mm. for the oxygen saturation pressure of lipoxidase and it was found that, under the assay conditions described here, bleaching rates were almost doubled when the reaction mixture was saturated with oxygen. However, the passage of a current of oxygen through the reaction mixture has the disadvantage of selectively removing acetone/ethanol from the system and thus rendering the carotene dispersion less stable. Moreover,

conditions of active oxygenation are not easy to reproduce. In the assay system described, sub-optimal but reproducible conditions are easily obtained if the reaction buffers are left overnight in the flasks to equilibrate with atmospheric oxygen. This procedure was therefore adopted as being convenient and reliable.

Effect of temperature.—Fig. 8 shows the effect of temperature on the carotene system at pH 6·3. The optimum of 25° is in agreement with that of other studies on carotene bleaching. The reaction rate is only slightly decreased at room temperature, and it is within the accuracy of the method to work at 20 ± 2°.

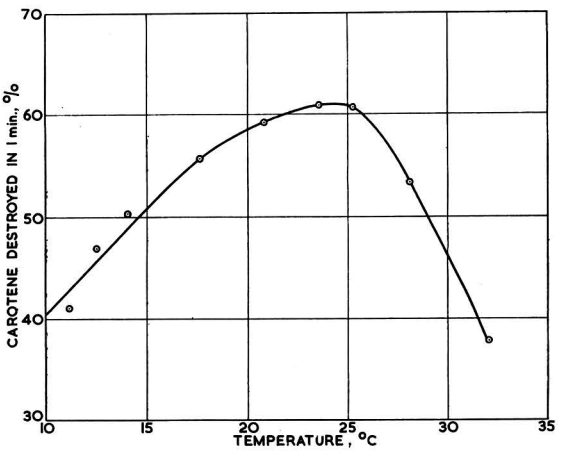

FIG. 8.—*Temperature/velocity curve at pH 6*

Effect of reaction time.—Fig. 9 shows the course of carotene destruction with time in the presence of about one unit of enzyme at pH 6. The flattish appearance of this curve indicates that small errors in timing the reaction would bear an almost linear relationship to carotene destruction.

Effect of enzyme concentration.—Fig. 10 illustrates the effect of concentration of enzyme on the reaction system under the conditions of the assay at pH 6. In the region of about 50% destruction of carotene the relationship is again almost linear.

Discussion

In the method described, an effort was made to achieve a system having adequate stability without the use of surface-active or dispersing agents as employed in previous assays. As shown by results not quoted here, such compounds, apart from their function of improving dispersion and stability, may have an inhibitory effect on bleaching, and this effect varies with their ratio to substrate. Strict adherence to procedure, coupled with short reaction times, gave reproducible results, despite the probability that substrate dispersion varies during the reaction period.

The use of caustic soda to stop the reaction eliminated troubles associated with those systems that use the measurement of time to half-bleach as an index

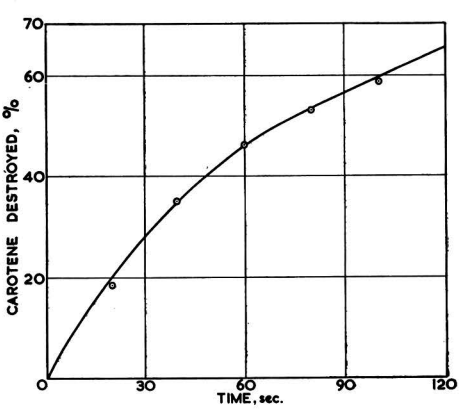

FIG. 9.—*Carotene destruction/time curve at pH 6*

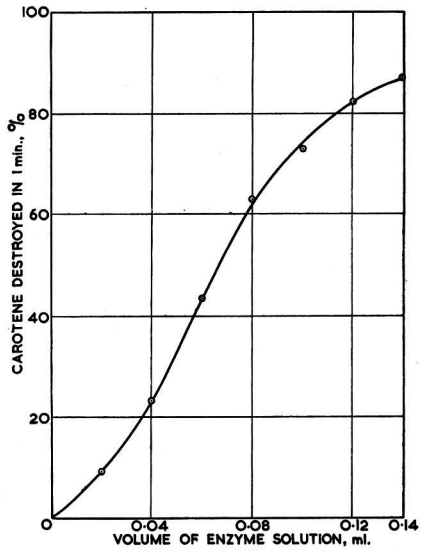

FIG. 10.—*Carotene destruction/enzyme concentration curve at pH 6*

of enzyme activity. Although short reaction times are desirable, in such systems bubbles disperse only slowly and give trouble with colour readings, and, in addition, there are turbidities due to protein or linoleate.

The necessity of having precise information on the effect of pH on the system is stressed by the recent work of Tappel.[24] In this work the restriction of fatty acid peroxidation by haem proteins to low pH values has been made a criterion of distinction between the fatty acid oxidase action of haem proteins and that of lipoxidase.

The use of carotene in lipoxidase assays has been criticized by Sumner & Smith[14] on the grounds that carotene solutions are unstable and that carotene soon separates from aqueous dispersions. Carotene solutions prepared by the method described above are stable over a period of months in their original solvent, and in the reaction system for many times the period required for the reaction. Difficulties do arise if the buffer strengths are exceeded and if the order of mixing of reactants is not strictly adhered to.

With due allowance for its limitations, the method has the advantage of speed where large numbers of analyses are required, and has been used not only for the analysis of soya extracts but also, with minor modifications, for the assay of lipoxidase activity in wheat germ and wheat flour. It may be of wider applicability than the recent manometric technique of Irvine & Anderson.[17] A future paper will report work on the application of this assay system to sources of lipoxidase other than soya.

Conclusions

1. A system for the study of the lipoxidase-catalysed bleaching of carotene in the presence of linoleate is described.

2. The effects of temperature, enzyme concentration, substrate concentration, time and pH on bleaching rates are discussed.

3. Evidence is presented that the interaction of linoleate and carotene dispersions is the principal factor determining the form of the pH–activity curve.

4. The system as described may be used to assay soya-bean lipoxidase. Its use, with minor modifications, may be extended to the measurement of lipoxidase activity in wheat products.

5. The rapidity of the method, the wide range of pH over which it may be used, and the simplicity of the equipment required renders the method particularly suitable for industrial control work.

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References

[1] Holman, R. T. & Bergstrom, S., ' The Enzymes ', 1951 (Eds.: Sumner, J. B. & Myrback, K.), Vol. II, Part 1, p. 559 (New York: Academic Press)
[2] Haas, L. W. & Bohn, R., *U.S.P.* 1,957,333–1,957,337 (1934)
[3] Hauge, S. M., *J. biol. Chem.*, 1935, **108**, 331
[4] Wilbur, J. W., Hilton, J. H. & Hauge, S. M., *J. Dairy Sci.*, 1935, **18**, 661
[5] Banks, A., *J. Soc. chem. Ind., Lond.*, 1944, **63**, 8
[6] Lea, C. H., *J. Soc. chem. Ind., Lond.*, 1937, **56**, 376T
[7] Watts, B. M. & Peng, D-H., *J. biol. Chem.*, 1947, **170**, 441
[8] Irvine, G. N. & Winkler, C. A., *Cereal Chem.*, 1950, **27**, 205
[9] Irvine, G. N. & Winkler, C. A., *Cereal Chem.*, 1951, **28**, 158
[10] Rank, J. & Hay, J. G., B.P. 646,311 (1951)
[11] Theorell, H., Holman, R. T. & Åkeson, Å., *Arch. Biochem.*, 1947, **14**, 250
[12] Reiser, R. & Fraps, C. S., *J. Ass. off. agric. Chem. Wash.*, 1943, **26**, 186
[13] Cosby, E. L. & Sumner, J. B., *Arch. Biochem.*, 1945, **8**, 259
[14] Sumner, J. B. & Smith, G. N., *Arch. Biochem.*, 1947, **14**, 87
[15] Balls, A. K., Axelrod, B. & Kies, M. W., *J. biol. Chem.*, 1943, **149**, 491
[16] Theorell, H., Bergstrom, S. & Åkeson, Å., *Pharm. Acta Helvet.*, 1946, **21**, 318
[17] Irvine, G. N. & Anderson, J. A., *Cereal Chem.*, 1953, **30**, 247
[18] Hawthorn, J. & Blain, J. A., unpublished data
[19] McIlvaine, T. C., *J. biol. Chem.*, 1921, **49**, 183
[20] McCutcheon, J. W., *Org. Synth.*, 1942, **22**, 75
[21] Holman, R. T., *Arch. Biochem.*, 1947, **15**, 403
[22] Smith, G. N. & Sumner, J. B., *Arch. Biochem.*, 1948, **19**, 89
[23] Fukuba, H., *J. agric. chem. Soc. Japan*, 1952, **26**, 170
[24] Tappel, A. L., *Food Res.*, 1953, **18**, 104

STUDIES ON EGG SHELLS. III.*—Some Physical and Chemical Characteristics of the Egg Shells of Domestic Hens

By C. TYLER and F. H. GEAKE

Fifty eggs have been examined and a number of physical and chemical characteristics determined. These include shell thickness, porosity coefficients, pore counts, membrane-, pore- and matrix-protein, calcium, carbonate, magnesium, phosphorus and citric acid. Apart from calcium, carbonate and magnesium, the characteristics all show pronounced bird differences. Certain relationships such as shell thickness : weight per unit area, shell weight : calcium content, calcium content : carbonate content and pore count : porosity coefficient have also been developed, and some may prove to be of value in eliminating laborious work such as shell-thickness measurements and calcium and carbonate determinations.

Introduction

In a previous paper Tyler & Geake[1] have pointed out how meagre are the data on the detailed chemical composition of egg shells, and in particular, those on the protein of egg shells. This paper is concerned with an attempt to obtain as many results as possible on each of a number of eggs so that the general body of information about certain physical and chemical characteristics of egg shells would be increased. At the same time it was thought that it might be possible to observe certain relationships between these characteristics.

Material

A batch of 50 hen eggs obtained over a period of 14 days from 14 different birds (Rhode Island Red × Light Sussex) (R.I.R. × L.S.) was used, but, owing to accidents, only 48 or 49 values are available. The lowest number of eggs from one bird was one, and the highest was five. The birds were kept in battery cages and fed on a normal ration plus supplements of different kinds of seaweed meal but, from the figures obtained, it was quite clear that no differences due to diet appeared. All the eggs were therefore treated as coming from the 14 different birds without any consideration as to differences in ration.

Methods

Eggs were collected and weighed as soon as possible after laying. The surface area, S, in sq. cm., was calculated from the fresh weight, W, in g., by the formula of Mueller & Scott,[2] namely: $S = 4{\cdot}67W^{2/3}$.

* Part II: *J. Sci. Fd Agric.*, 1953, **4**, 266

The porosity coefficient of each egg was then measured by the method of Tyler.[3] After this determination, the eggs were candled and a description of their candling appearance was noted.

The eggs were then broken and the contents removed, the two portions of shell being carefully washed on the inside, dried and weighed.

Membrane-, pore- and matrix-protein were next determined on the shells by the method described by Tyler & Geake.[1] Other samples of true shell, i.e. shell from which the membranes had been removed by boiling in a 2·5% solution of sodium hydroxide for 5 minutes, were used for (*a*) measurement of shell thickness (average of five readings); (*b*) pore count, as described by Tyler;[4] and (*c*) chemical composition.

Under heading (*c*) were included determinations of calcium,[5] phosphorus (Koenig & Johnson,[6] as modified by Kitson & Mellon[7]), magnesium,[8] citric acid (Weil-Malherbe & Bone,[9] as modified by Taylor[10]) and carbonate.[11]

Many of the results were subjected to an analysis of variance suited to the needs of data in which sub-samples are of unequal size, thus enabling comparisons to be made 'between' birds and 'within' birds, i.e. to consider if there were significant differences between bird means.

Not all the results are reported in this paper, such values as surface area, shell weight, shell thickness and candling appearances being omitted, although some have been used in calculations embodied in the paper.

Results

Shell thickness

It is well known that birds show considerable differences in shell thickness and therefore the data which bear this out have not been presented here. However, one interesting relationship has appeared.

Thickness was measured with a micrometer screw gauge and the mean of five readings taken. The five readings were often variable and coefficients of variation ranged from 0·2 to 6·6. Since this process of measurement is lengthy and tiresome, it was decided to investigate other means of obtaining a measure of thickness. It was found that the regression is highly significant ($P < 0{\cdot}001$) between thickness and true shell-weight per sq. cm.

$$Y = 4{\cdot}83X - 15{\cdot}8$$

where X is wt. of true shell in mg. per sq. cm. and Y is the thickness in μ.

The use of such an equation for hen eggs would give a reasonably accurate and much quicker measure of mean shell-thickness and, in manipulation, would only involve removing the membrane, which must be removed in any case, when measuring shell thickness. Clearly, this particular equation refers only to normal shells but it may be found that it would apply to quite thin ones. Another equation might be developed for other species, but it would probably be not very different from this one.

Pore count

The pore count is expressed as the number of pores per sq. cm. of shell. From Table I it is quite clear that birds show differences in pore counts between themselves. Such differences are highly significant ($P < 0{\cdot}001$). The lowest mean count was 107 for bird 2, and the highest, 227 for bird 9, and there were no aberrant counts for individual eggs.

Since pore counting was done by the method of Tyler[4] it follows that a frequency distribution was also obtained of the readings 0, 1, 2, 3, . . . n pores per sq. mm. This will be discussed in a further paper.

When calculated in terms of total pores (results not given), the lowest mean value is 7670 for bird 2 and the highest 16,403 for bird 8. The fact that these values are, in general, much higher than those noted by the other workers has already been discussed by Tyler.[4]

Porosity coefficients

The coefficients given in Table II are based on the rate of loss in weight, in mg. per sq. cm., of shell area per day at 15° over calcium chloride in a standard-size desiccator. Once again there are highly significant differences between birds ($P < 0{\cdot}001$). If birds 2 and 12 are ignored, for obvious reasons, the lowest mean value is 1·53 for bird 13 and the highest is 2·31 for bird 6. There were two aberrant results, namely a value of 0·65 for bird 2 and one of 0·44 for bird 5, but as far as could be seen there were no other characteristics that marked out these two eggs as being exceptional. Certainly, neither the outward appearance nor the candling appearance of these two eggs gave any indication of their very low porosity.

Table I

Pore count as number of pores per sq. cm.

	Birds													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	166	101	154	185	136	139	140	197	221	136	136	181	121	141
	144	112	181	162	150	128	156	230	232	132	135		105	134
	155		168	155	172	165		213		141	144		109	137
			157	193	122	144		237		142			120	150
				175	144					112				
Bird means	155	107	165	174	145	144	148	219	227	133	138	181	114	141

General mean 154

Analysis of variance

	Degrees of freedom	Sum of squares	Variance	Variance ratio
Between birds	13	48600	3738	33·98***
Within birds	34	3755	110	
Total	47	52355		

In this and succeeding Tables, *, ** and *** indicate significant at $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively

Table II

Porosity coefficient based on rate of loss of weight in mg./sq. cm./day at 15° over calcium chloride

	Birds													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	1·91	0·65	1·99	2·12	2·02	2·13	2·16	2·34	2·24	1·93	2·30	2·42	1·42	1·91
	1·27	1·57	1·99	1·57	2·08	2·04	1·87	2·32	2·18	1·71	2·16		1·58	2·01
	1·69		1·83	1·55	1·84	2·66		2·10		1·73	1·98		1·41	2·10
	1·58		1·88	1·64	1·68	2·42		2·42		1·43			1·72	1·92
				1·56	0·44					1·48				
Bird means	1·61	1·11	1·92	1·69	1·61	2·31	2·02	2·30	2·21	1·66	2·15	2·42	1·53	1·99

General mean 1·86

Analysis of variance

	Degrees of freedom	Sum of squares	Variance	Variance ratio
Between birds	13	4·9690	0·3822	3·99***
Within birds	35	3·3554	0·0959	
Total	48	8·3244		

Almquist & Holst[12] showed that there was a high degree of correlation between loss in weight and a semi-quantitative value for pore count, and it is natural to expect that there may be some relationship between pore count, X, and porosity coefficient, Y. This was therefore tested with the 14 available bird means. It was found that the regression was significant at $P = 0.05$ and just failed to be so at $P = 0.01$. Thus there is a significant relationship between the two factors. The linear equation is:

$$Y = 0.0069X + 0.81$$

If it is assumed that this equation can be extended beyond the range of the present data, then with no pores there would be a loss of water vapour from the egg, and the magnitude of the independent term gives the minimum porosity coefficient, i.e. for a shell with no pores the porosity coefficient is 0·81. This value is very large and most unlikely. It may therefore be suggested that over a wider range of results covering fewer pores per sq. cm. a linear relationship might not hold. However, it cannot be assumed that the curve would necessarily pass through the origin, since there is no evidence that the shell apart from the pores is completely impervious. In addition it must be remembered that there are other factors that

influence porosity, such as the size of cross section of the pores and possibly their length, which in turn will be influenced by the thickness of the shell.

It is also of interest to consider the porosity coefficient per pore. The results are given in Table III. The ordinary porosity coefficient is measured in mg., but the porosity coefficient per pore has been expressed in μg. If the porosity coefficient per pore is proportional to the size of either area or diameter of the pore, then it is clear from these results that birds 8 and 9 show a high porosity because of a large number of pores of small size. Bird 6, on the other hand, has a large porosity because it has a smaller number of large pores. This is also confirmed by the results in Table VII. Thus, it would appear that both pore numbers and pore size influence porosity as measured by water loss, a result one would expect.

Table III

Porosity coefficient per pore, μg.

	Birds													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	7·7	6·4	12·9	11·5	14·9	15·3	15·4	11·9	10·1	14·2	16·9	13·4	11·7	13·5
	11·7	14·0	11·0	9·7	13·9	15·9	12·0	10·1	9·4	13·0	16·0		15·0	15·0
	10·2		10·9	10·0	10·7	16·1		9·9		12·3	13·8		12·9	15·3
			12·0	8·5	13·8	16·8		10·2		10·1			14·3	12·8
				8·9	3·0					13·2				
Bird means	9·9	10·2	11·7	9·7	11·3	16·0	13·7	10·5	9·8	12·6	15·6	13·4	13·5	14·2

General mean 12·2

Membrane protein

This determination probably includes the cuticle, as pointed out by Tyler & Geake,[1] and, on the basis of relative thickness of the two, this will tend to increase membrane values by about 10%. The relative error will not, however, be as big as this because all values are probably increased to approximately the same extent.

Romanoff & Romanoff[13] give the estimated composition of the membrane as: water 20·0, protein 70·0 and inorganic matter 10·0%. They do not state how the membranes were removed for analysis nor whether the 10% of ash includes the sulphur of the keratin. The present authors prefer to regard the membrane as pure protein, and have therefore not modified their values in any way to allow for other possible constituents, although if this were done it would again only affect the absolute and not the relative values between birds.

It was realized, however, that the surface area of the egg, which can here be regarded as equal to the inner surface area of the shell covered by membrane, would influence the amount of membrane. Therefore all results have been expressed in terms of membrane per sq. cm., and these are shown in Table IV.

Table IV

Weight of membrane (plus cuticle), mg. per sq. cm.

	Birds													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	4·78	3·51	3·15	4·29	3·75	4·32	4·78	3·72	4·79	4·10	4·51	4·48	3·37	4·28
	4·64	3·03	3·00	5·30	3·59	4·08	3·82	4·07	3·51	3·81	4·55		3·20	4·32
	4·57		3·33	4·50	3·69	4·10		4·11		4·14	4·37		3·19	4·19
	4·47		2·94	4·01	3·69	3·98		3·92		3·91			3·27	4·15
				4·40	3·76					3·96				
Bird means	4·62	3·27	3·11	4·50	3·70	4·12	4·30	3·96	4·15	3·98	4·48	4·48	3·26	4·24

General mean 3·99

Analysis of variance

	Degrees of freedom	Sum of squares	Variance	Variance ratio
Between birds	13	11·117	0·855	10·8***
Within birds	35	2·776	0·079	
Total	48	13·893		

There are marked significant differences between birds ($P < 0.001$); the lowest value is 3·11 mg. for bird 3 compared with the highest value of 4·62 for bird 1.

If the amount of water in the membrane is taken as 20% and the density of wet membrane as 1·005, then the thickness can be calculated. If the general mean of 4·0 mg. of membrane per sq. cm. is used, then a thickness of 0·050 mm. is obtained, compared with the values of 0·050 mm. for a bantam and 0·065 mm. for a Leghorn, given by Romanoff & Romanoff.[13] These workers suggest that membrane thickness is roughly proportional to the size of the egg and, since the R.I.R. × L.S. cross.is bigger than the Leghorn and much bigger than the bantam in size, it appears as if our figures are much smaller than those quoted. They are also smaller than the values given by Moran & Hale,[14] namely 0·070–0·084 mm. for hen eggs. It is, however, important to recognize that Moran & Hale fixed and stained their preparations before measurement, and that Romanoff & Romanoff state that various values have been observed for the thickness probably because of the shrinkage or swelling of the membranes under different treatments. It may therefore be that the weight of protein per unit area is just as good a guide to the average membrane-thickness as is actual measurement of the thickness of isolated pieces of treated membrane. The present authors realize that their value would be even better if the cuticle protein could be determined separately. Such a determination is not impossible, but no method suitable for routine work has yet been found.

Shell protein

The protein content of the true shell, which remains after the removal of membrane and probably the cuticle, is of some interest. The results are given in Table V and are expressed as percentage values. The differences between bird means are highly significant ($P < 0.001$) despite the fact that the whole set of 49 values lies between 1·31 and 1·73. With some of the birds the values are remarkably constant, e.g. birds 6 and 10.

Table V

Total shell protein, per cent.

	Birds													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	1·42	1·31	1·50	1·52	1·42	1·69	1·44	1·46	1·58	1·38	1·64	1·71	1·48	1·53
	1·41	1·39	1·55	1·59	1·44	1·72	1·53	1·63	1·58	1·40	1·58		1·50	1·54
	1·48		1·61	1·68	1·56	1·71		1·48		1·42	1·68		1·53	1·43
	1·53		1·51	1·50	1·51	1·69		1·45		1·42			1·55	1·64
				1·73	1·44					1·42				
Bird means	1·46	1·35	1·54	1·60	1·47	1·70	1·49	1·51	1·58	1·41	1·63	1·71	1·52	1·54

General mean 1·53

Analysis of variance

	Degrees of freedom	Sum of squares	Variance	Variance ratio
Between birds	13	0·3984	0·0306	8·01***
Within birds	35	0·1338	0·0038	
Total	48	0·5322		

Almquist[15] gives values for shell protein, which he calls matrix protein, of 1·27–3·39, with a mean of 1·98%. However, his method for obtaining the protein was entirely different from ours.

Pore protein

The pore protein has been expressed as a percentage of the true shell, and the values are given in Table VI. The analysis of variance once more shows a highly significant difference ($P < 0.001$) between birds, mean values ranging from 0·61 to 0·91 if the single value for bird 12 is ignored.

A relationship has already been shown to exist between pore count and porosity coefficient, and some comment has been made on pore size. Pore protein should also give some information about pore size, and in Table VII the amount of pore protein (μg.) per pore is shown. Again there are highly significant differences ($P < 0.001$) between birds and a very considerable range of values from 1·90 to 6·31. These figures represent the average amount of protein in a pore but it is possible to obtain some idea of their shape from the results.

Table VI

Pore protein, per cent.

	Birds													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	0·72	0·64	0·72	0·74	0·61	0·93	0·65	0·53	0·74	0·77	0·83	1·07	0·86	0·64
	0·68	0·70	0·87	0·83	0·65	0·99	0·79	0·74	0·78	0·71	0·71		0·83	0·67
	0·77		0·84	0·93	0·73	0·87		0·60		0·81	0·83		0·90	0·66
	0·82		0·75	0·75	0·72	0·85		0·57		0·81			0·93	0·79
				1·01	0·67					0·71				
Bird means	0·75	0·67	0·80	0·85	0·68	0·91	0·72	0·61	0·76	0·76	0·79	1·07	0·88	0·69

General mean 0·77

Analysis of variance

	Degrees of freedom	Sum of squares	Variance	Variance ratio
Between birds	13	0·4538	0·0349	6·84***
Within birds	35	0·1795	0·0051	
Total	48	0·6333		

Table VII

Protein per pore, μg.

	Birds													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	3·39	3·86	3·57	2·90	3·38	5·17	3·49	1·83	2·03	4·26	4·59	4·56	5·98	2·90
	4·53	4·04	3·59	3·73	3·09	5·97	3·94	2·18	2·14	3·92	3·92		6·37	3·14
	4·53		3·77	4·43	3·09	3·98		2·03		4·41	4·27		6·52	3·17
			3·64	2·94	4·49	4·00		1·57		4·31			6·35	3·40
				4·12	3·36					4·05				
Bird means	4·15	3·95	3·64	3·62	3·48	4·78	3·72	1·90	2·09	4·19	4·26	4·56	6·31	3·15

General mean 3·85

Analysis of variance

	Degrees of freedom	Sum of squares	Variance	Variance ratio
Between birds	13	53·9326	4·148	17·7***
Within birds	34	7·9558	0·234	
Total	47	61·8884		

Romanoff & Romanoff[13] state that the pores are oval and give values for the two axes for the largest and smallest pores. If these are averaged and converted to figures for circular pores the value is 9 μ for the mean radius of pores in hen eggs. Haines & Moran,[16] however, give values of 6·5 μ and 3 μ for the top and bottom radii respectively of a pore. Now the volume of pore protein per pore in a pore of radius 9 μ and length of 0·35 mm., i.e. the approximate thickness of a shell, will be $\pi \times (9 \times 10^{-3})^2 \times 0{\cdot}35$ cu. mm. $= 89{\cdot}08 \times 10^{-6}$ cu. mm. If the protein has a density of about 1·00, as it will have in the wet state, then this represents $89{\cdot}08 \times 10^{-6}$ mg. $= 89{\cdot}08 \times 10^{-3}$ μg. of protein, i.e. about 0·09 μg. This result was obtained by using 9 μ for the radius of a pore; if a lower value had been used, e.g. that of Haines & Moran, the amount of protein would have been considerably smaller. Now in Table VII the general mean value for protein per pore is given as 3·85 μg., hence the pores measured by Romanoff & Romanoff would contain approximately only $(0{\cdot}09/3{\cdot}85) \times 100 = 2{\cdot}3\%$ of the protein as measured by us.

Marshall & Cruickshank[17] have drawn attention to the plaque at the mouth of each pore and they maintain that this is the true evaporating surface. Romanoff & Romanoff agree with this and give a diagram showing that the pore has a shape rather like a mushroom with a very thin stalk and wide but thin head. If this is correct then the measurements of pore diameter presumably refer to the 'stalk' of the mushroom and not to the 'head' or plaque. In our method, the protein in the whole pore, including the head, will have been determined

and this probably accounts for the large discrepancy. Clearly, more information is needed on pore shape and size before attempts can be made to correlate pore protein with porosity.

Matrix protein

The results of matrix protein, expressed as a percentage of the true shell, are given in Table VIII. Again, there are highly significant differences between birds ($P < 0.001$).

Table VIII

Matrix protein, per cent.

	Birds													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	0·70	0·67	0·78	0·78	0·81	0·76	0·79	0·93	0·84	0·61	0·81	0·64	0·62	0·89
	0·73	0·69	0·68	0·76	0·79	0·73	0·74	0·89	0·80	0·69	0·87		0·67	0·87
	0·71		0·77	0·75	0·83	0·84		0·88		0·61	0·85		0·63	0·77
	0·71		0·76	0·75	0·79	0·84		0·88		0·61			0·62	0·85
				0·72	0·77					0·71				
Bird means	0·71	0·68	0·75	0·75	0·80	0·79	0·77	0·90	0·82	0·65	0·84	0·64	0·64	0·85

General mean 0·76

Analysis of variance

	Degrees of freedom	Sum of squares	Variance	Variance ratio
Between birds	13	0·3058	0·0235	17·9***
Within birds	35	0·0460	0·0013	
Total	48	0·3518		

It is of interest to note that between pore protein, per cent., X, and matrix protein, per cent., Y, the regression is significant at $P = 0.05$.

The equations are: $Y = 1{\cdot}08 - 0{\cdot}39X$

and: $X = 1{\cdot}35 - 0{\cdot}76Y$

Thus, as one value increases the other decreases, but not at an equivalent rate, hence the total protein in the true shell is not constant.

Calcium and carbonate

It was shown by Tyler[18] that the percentage of calcium in the nitrogen-free shell is practically constant. The figures presented in Table IX support this and, as was to be expected, there are no significant differences between bird means.

Table IX

Calcium, per cent., in nitrogen-free shell

	Birds													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	39·3	38·6	38·3	38·8	38·1	39·3	38·8	38·6	38·8	38·5	38·1	38·3	39·1	39·0
	38·8	38·6	39·0	38·1	38·7	38·9	38·0	38·6	38·7	38·6	38·3		39·2	39·1
	38·1		38·9	39·2	38·3	38·6		38·4		38·6	38·7		38·6	38·5
	38·3		38·7	38·3	38·7	38·8		39·0		38·4			39·0	38·2
				38·9	39·1					38·8				
Bird means	38·6	38·6	38·7	38·7	38·6	38·9	38·4	38·7	38·8	38·6	38·4	38·3	39·0	38·7

General mean 38·7

Analysis of variance

	Degrees of freedom	Sum of squares	Variance	Variance ratio
Between birds	13	1·266	0·097	0·78 N.S.
Within birds	35	4·352	0·124	
Total	48	5·618		

In this and succeeding Tables, N.S. indicates not significant

In the same paper[18] a highly significant correlation was found to exist between shell weight (including membranes), Y, and total shell calcium, X. The regression equation with Y as the independent variable was given in that paper, but for reasons to be made clear later the other equation is preferable here. This has been calculated and is:

$$X = 0{\cdot}386Y - 0{\cdot}112 \quad (73 \text{ shells})$$

Corresponding results were available in the present experiment. For 49 shells the regression was highly significant ($P < 0{\cdot}001$). The equation is:

$$X = 0{\cdot}374Y - 0{\cdot}072$$

The regression coefficients in these two equations are not significantly different and their close agreement can be seen more clearly by the following figures. For a shell of 3 g. weight the calcium content calculated from the two equations is found to be 1·046 and 1·050 g. respectively, and for one of 6 g. weight it is 2·204 and 2·172 g. respectively.

It would thus appear that much time and labour could be saved in certain poultry experiments, particularly mineral-balance experiments where absolute accuracy can never, in any event, be achieved, by calculating the amount of calcium in the shell from the dry weight of shell plus membranes. Care should be taken, however, not to use the equation beyond the range of shell weights used in calculating it. The first equation given here covers shell weights from 2·94 to 6·44 g. and it is unlikely that there will be many shells outside this range.

No doubt the equation would be more valuable if the true shell weight was used, but this would necessitate the removal of the membrane, thus losing much of the time saved on the calcium determination.

Table X gives the values for carbonate, as CO_2, but no analysis of variance has been made since the figures for carbonate run closely parallel to those for calcium. Tyler[18] showed that the weight of calcium, X, is related to the weight of carbonate, Y, by the highly significant ($P < 0{\cdot}001$) regression equation

$$Y = 1{\cdot}118X - 0{\cdot}0018$$

The present set of results also gives an equally significant regression:

$$Y = 1{\cdot}117X - 0{\cdot}015$$

Table X

Carbonate, per cent., as CO_2, in nitrogen-free shell

	Birds													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	42·8	42·5	43·4	42·9	42·7	43·1	42·9	41·9	42·6	42·6	41·7	43·1	43·6	41·6
	43·2	42·7	42·9	43·0	42·5	43·4	43·0	42·9	43·6	42·8	42·0		43·5	42·9
	42·8		42·8	43·3	43·0	42·4		43·0		42·0	43·0		43·3	43·3
	42·6		42·4	43·3	43·3	43·0		43·3		41·9			42·7	43·5
				42·7	43·7					44·0				
Bird means	42·9	42·6	42·9	43·0	43·0	43·0	43·0	42·8	43·1	42·7	42·2	43·1	43·3	42·8

General mean 42·9

There is no significant difference between the regression coefficients and, from these two equations, the calculation of carbonate corresponding to 2 g. of calcium gives 2·234 and 2·219 g. respectively, i.e. less than 1% error. Within the range of shells studied in this paper, there is always more carbonate than is required to form calcium carbonate. This agrees with the results from the earlier equation and supports the idea that some of the carbonate must be combined with magnesium.

The difference between the independent terms in these two equations may be due to the fact that carbonate was determined in the first experiment by means of the Collins calcimeter[19] whereas in this experiment the determination was by a titration method.

Magnesium, phosphorus, and citric acid

Table XI gives the figures for the percentage of magnesium in the nitrogen-free shell. The values are more variable than those for calcium, but there were no significant differences between birds. Some of the variability may be due to the difficulty of determining such small quantities of magnesium in the presence of large amounts of calcium.

Table XI

Magnesium, per cent., in nitrogen-free shell

	Birds													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	0·59	0·72	0·64	0·58	0·51	0·54	0·56	0·57	0·57	0·58	0·56	0·58	0·58	0·35
	0·69	0·56	0·60	0·66	0·59	0·59	0·50	0·49	0·56	0·66	0·47		0·59	0·55
	0·64		0·56	0·60	0·56	0·58		0·55		0·62	0·63		0·57	0·59
			0·58	0·66	0·58	0·56		0·63		0·64			0·65	0·63
				0·59	0·58					0·63				
Bird means	0·64	0·64	0·60	0·62	0·56	0·57	0·53	0·56	0·57	0·63	0·55	0·58	0·60	0·53

General mean 0·59

Analysis of variance

	Degrees of freedom	Sum of squares	Variance	Variance ratio
Between birds	13	0·0579	0·004454	1·36 N.S.
Within birds	34	0·1115	0·003279	
Total	47	0·1694		

Table XII shows the relevant results from the determinations of phosphorus and there are significant differences ($P < 0·001$) between bird means; the lowest value is 0·11% and highest 0·20%. A difference in analytical methods should be noted here. In the earlier work, which gave the values quoted by Tyler,[18] shells were analysed with membrane and cuticle attached, but in the present work the analyses were made on shells devoid of membrane and cuticle; both sets of values for phosphorus content are, however, very similar.

Table XII

Phosphorus, per cent., in nitrogen-free shell

	Birds													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	0·11	0·19	0·17	0·13	0·13	0·13	0·13	0·12	0·14	0·17	0·11	0·17	0·11	0·12
	0·10	0·20	0·13	0·11	0·13	0·11	0·12	0·12	0·10	0·14	0·11		0·12	0·12
	0·11		0·12	0·12	0·10	0·15		0·11		0·17	0·11		0·11	0·13
	0·10		0·14	0·11	0·14	0·12		0·11		0·15			0·12	0·13
				0·10	0·13					0·09				
Bird means	0·11	0·20	0·14	0·11	0·13	0·13	0·13	0·12	0·12	0·14	0·11	0·17	0·12	0·13

General mean 0·13

Analysis of variance

	Degrees of freedom	Sum of squares	Variance	Variance ratio
Between birds	13	0·01815	0·00140	5·2***
Within birds	35	0·00933	0·00027	
Total	48	0·02748		

Citric acid has been found in most tissues, including bones and teeth, and in egg shells. It was therefore decided to obtain figures on this substance. These are presented in Table XIII. The analysis of variance once more shows significant differences ($P < 0·001$) between bird means. It is of interest to consider where the citric acid occurs in the shell. The analysis, carried out on true shell, indicates the presence of some citric acid here, but its occurrence in the membrane as well is not ruled out. The regression of mg. of citric acid, Y, on mg. of total shell-protein, X, was highly significant ($P < 0·001$) and the equation is:

$$Y = 0·09X - 1·56$$

The evidence therefore suggests that the citric acid may be in some way associated with the shell protein, but it is not yet possible for us to state whether it is associated with all the shell protein or only with the pore- or matrix-protein. Since citric acid is present in tissue fluids and secretions it is not surprising if some becomes locked up in the body of the shell during the deposition of protein and mineral matter.

Table XIII

Citric acid, per cent., in nitrogen-free shell

	Birds													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	0·12	0·09	0·09	0·10	0·13	0·11	0·10	0·12	0·11	0·09	0·12	0·12	0·10	0·08
	0·10	0·07	0·07	0·15	0·10	0·13	0·10	0·13	0·10	0·12	0·11		0·13	0·11
	0·10		0·08	0·12	0·15	0·14		0·11		0·10	0·10		0·12	0·07
			0·10	0·11	0·12	0·13		0·09		0·12			0·12	0·09
				0·15	0·11					0·11				
Bird means	0·11	0·08	0·09	0·13	0·12	0·13	0·10	0·11	0·11	0·11	0·11	0·12	0·12	0·09

General mean 0·11

Analysis of variance

	Degrees of freedom	Sum of squares	Variance	Variance ratio
Between birds	13	0·01007	0·000775	3·18**
Within birds	34	0·00830	0·000244	
Total	47	0·01837		

Discussion

It is evident that, of the shell characteristics studied, with the exception of calcium, carbonate and magnesium content, all are indicative of the individuality of the bird and that highly significant differences are to be found between bird means. This does not necessarily indicate that each bird mean is different from all the others, but that the birds may fall into groups although the grouping need not be the same for each characteristic. These findings, though not surprising, are of great interest, and encourage an even more detailed study of larger numbers of eggs.

The expression of shell thickness and amount of membrane, each in terms of weight per unit area, has obvious advantages. Similarly, equations relating shell weight to calcium content and the latter to carbonate content should certainly minimize the work required in certain experiments, but care must be taken not to apply such relationships to other species until they have been firmly established.

The prospect of establishing a relationship between evaporating area and porosity is of interest, but it is of particular importance to try and ascertain more clearly the actual size and shape of the pores by microscopic studies, if this line is to prove fruitful.

The relationship shown to exist between citric acid and shell protein should also prove worthy of more detailed study.

Finally, despite the arbitrary nature of some of the methods employed, it appears that they give results that can be regarded with some confidence, for they clearly distinguish between eggs from different birds.

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References

[1] Tyler, C. & Geake, F. H., *J. Sci. Fd Agric.*, 1953, **4**, 261
[2] Mueller, C. D. & Scott, H. M., *Poult. Sci.*, 1940, **19**, 163
[3] Tyler, C., *J. agric. Sci.*, 1945, **35**, 168
[4] Tyler, C., *J. Sci. Fd Agric.*, 1953, **4**, 266
[5] Wheatley, V. R., *Analyst*, 1944, **69**, 207
[6] Koenig, R. A. & Johnson, C. R., *Industr. Engng Chem. (Anal.)*, 1942, **14**, 155
[7] Kitson, R. E. & Mellon, M. G., *Industr. Engng Chem. (Anal.)*, 1944, **16**, 379
[8] Hunter, J. G., *Analyst*, 1949, **75**, 91
[9] Weil-Malherbe, H. & Bone, A. D., *Biochem. J.*, 1949, **45**, 377
[10] Taylor, T. G., *Biochem. J.*, 1953, **54**, 48
[11] Tinsley, J., Taylor, T. G. & Moore, J. H., *Analyst*, 1951, **76**, 300
[12] Almquist, H. J. & Holst, W. F., *Hilgardia*, 1931, **6**, 61
[13] Romanoff, A. L. & Romanoff, A. J., 'The Avian Egg', 1949, 1st edn. (New York: John Wiley and Sons Inc.)
[14] Moran, T. & Hale, H. P., *J. exp. Biol.*, 1936, **13**, 35
[15] Almquist, H. J., *Poult. Sci.*, 1934, **13**, 375
[16] Haines, R. B. & Moran, T., *J. Hyg., Camb.*, 1940, **40**, 453
[17] Marshall, W. & Cruickshank, D. B., *J. agric. Sci.*, 1938, **28**, 24
[18] Tyler, C., *Brit. J. Nutr.*, 1950, **4**, 112
[19] Collins, S. H., *J. Soc. chem. Ind., Lond.*, 1906, **25**, 518

CHANGES IN THE pH AND BUFFERING CAPACITY OF FISH DURING SPOILAGE

By C. L. CUTTING

Claims have recently been made once again for a test for freshness of fish based on a decrease in buffering capacity with increasing staleness. This decrease has been shown to be due chiefly to the bacterial reduction of trimethylamine oxide. However, under commercial conditions the results appear to be too irregular for such a test to be reliable. The buffering capacity of fish flesh in various ranges of pH and its variation with degree of spoilage have been almost completely accounted for by the contributions of proteins and water-soluble constituents. The pH changes in fresh and spoiling fish, which depend in large measure on buffering capacity, are also too irregular to be satisfactorily correlated with freshness.

Introduction

No objective tests for the freshness of fish have yet replaced sensory examination.[1, 2] In sea fish the production of amines by the bacterial reduction of trimethylamine oxide, a minor constituent, is apparent to the senses, and can be detected chemically before the appearance of products of incipient bacterial and autolytic breakdown of protein, such as ammonia, hydrogen sulphide and indole. The results of chemical estimation of water-soluble and volatile constituents, present only in small amounts, are irregular owing to individual and seasonal variations in initial chemical composition and bacterial flora and to variable losses under commercial conditions of storage in melting ice.[3]

Among physicochemical methods, the determination of pH has been much investigated. The pH of the flesh of freshly killed fish and shell-fish is usually close to 7·0, falling within a few hours of death, when *rigor mortis* has set in, to between 6·1 and 6·9;[4, 5] that of flat fish is perhaps slightly lower than that of round fish.[6–8] The amines and ammonia produced in stale fish result in an increase in pH, which has been investigated as a test for the freshness of whole fish[9] and fillets.[10] In the most recent work[11, 12] it is concluded that such tests are unreliable.

Notevarp & Hjorth-Hansen[13] noticed, in titrating fish-muscle press juice, that buffering capacity was lower in staler fish. Stansby & Lemon[14] devised a freshness test for haddocks based on this effect. They designated the volume (ml.) of N/60-acid required to reduce 5 g. of fish muscle from its initial pH to 6·0 '*B*', and that for a reduction from pH 6·0 to 4·3 '*A*'. They regarded the increase in *B* with staleness as an index of bacterial decomposition and attributed the reduction in *A* to autolysis of the protein. The freshness of fish was assessed by striking a balance between these two values. Griffiths & Stansby[15] introduced into the index of freshness the logarithm of the bacterial count. Fitzgerald & Conway[16] put the method on a statistical basis 'to enable sale price to be based on quality'. Stansby & Fitzgerald[17] devised apparatus for routine, automatic titration of ten samples simultaneously. Cutting[18, 19] associated the decrease in *A* with the bacterial breakdown of trimethylamine oxide, which exerts its maximum buffering effect at pH 4·6, to trimethylamine. The results were quite different from those of Stansby & Lemon[14] and not at all reproducible. Moreover, hydrolysis of fish muscle and fish myosin resulted, as anticipated by Beatty & Gibbons,[20] in an increase, rather than a decrease, in buffering capacity, and pieces of cod muscle stored under sterile conditions suffered no significant change in buffering capacity. Nickerson & Proctor[21] had concluded that sterility did not affect post-mortem buffering changes in haddock, although their results were conflicting, perhaps owing to the use of frozen fish, which are liable to lose soluble buffering constituents in the drip on thawing. Collins, Küchel & Beatty[22] found that the initial fall in the buffering capacity of cod-muscle press juice stored at 2° and 10° was followed by an increase when it was putrid, which was attributed to bacterial hydrolysis of the muscle proteins. Hjorth-Hansen,[23] using a somewhat different titration technique with cod stored at − 0·5°, also concluded that the method was unreliable as a test for freshness. However, Hacker[24] has recently reinvestigated the method, finding that fresh-water fish, which should contain no trimethylamine oxide, and cod had about the same initial buffering capacity, which on storage in air at 4° and 10° actually increased. She also tried to correlate the *A* and *B* values with organoleptic rating. She confirmed the observation that acid hydrolysis increased the buffering capacity of protein preparations. The following results were obtained in a more systematic reconsideration of the value of the titration test for fish stored in melting ice under the actual conditions normally employed commercially.

Experimental

Marine, demersal species caught by trawl net close to Aberdeen were gutted and washed at once, stowed in boxes and kept covered with melting ice. This treatment keeps fish really fresh for about 7 days, after which they deteriorate until they verge on inedibility after 14 to 16 days.[25] Herrings caught by commercial drifters were iced ungutted. Fresh-water species, taken from lakes by means of a seine net, were gutted, washed and iced. Sterile samples of fish flesh, crabs and the flesh of warm-blooded animals for comparison were stored in air at 0°

Several specimens, usually six to ten, were thoroughly mixed and minced for a sample. The experimental procedure of Stansby & Lemon[14] was slightly modified. Samples (5 g.) of flesh were minced in a Latapie mincer and dispersed in 100 ml. of distilled water. The pH of this suspension after additions of N/60-hydrochloric acid or sodium hydroxide was determined at 20° by means of a glass electrode and valve potentiometer. The quinhydrone electrode gave consistent readings with fresh fish but was unreliable with stale fish, since it is affected by traces of amines and is unstable above about pH 7·5.

Once titrating liquid had been added, the pH invariably drifted back, rapidly at first, and then more slowly, presumably owing to the mechanical obstruction of pieces of muscle to the penetration of ions. Mechanical stirring accelerated the initial drift, but the subsequent slow drift continued even if the suspension was left for hours after each fresh addition.

When solutions of extractives, 'actomyosin' suspension extracted by the method of Edsall,[26] or muscle ultra-filtrates were titrated there was no drift. Drift was unaffected by the degree of dilution and the strength of titrating liquid, or by variation of temperature between 0° and 20°.

The muscle suspension was titrated in steps to bring the pH to certain fixed values by rapid additions initially, followed by subsequent small additions to counteract the rapid drift until 'relative equilibrium' had been attained. The fixed pH values were 6·0, 5·3, 4·65 and 4·3 with acid, and 7·0, 8·0, 9·0 and 9·5 or 10·0 with alkali. Outside this range the effect of dilution was significant when N/60-solutions with 100-ml. samples were used. The titration with acid or alkali took about half an hour.

Following Bate-Smith,[27] buffering capacity is expressed as the 'buffering power', β, of van Slyke,[28] defined as $db/d(pH)$, where db is the amount of base in mg.-equivalents required per 100 g. of muscle to produce an increment d(pH) in pH. Values for β are convertible into A and B values.

Drift precluded an accurate titration curve to which tangents could be drawn in order to derive values of β at various pH values for whole muscle. Average values over certain ranges of pH were therefore calculated. Titrations were not reproducible to within $\pm$ 0·5 ml., so that individual values were not reliable to within $\pm$ 5%.

Hjorth-Hansen[23] overcame drift by a separate titration for each point of the curve. However, it is doubtful whether, in fact, he obtained true equilibrium even by this technique, because the pH of his suspension was higher than that of the supernatant liquid, which, again, was higher than that of the filtrate.

In addition to the determination of the initial pH of the muscle suspension some measurements were also made of the post-mortem pH changes of fish flesh *in situ*, by means of a probe glass electrode.

Results and discussion

Since the pH of fish muscle is determined by the buffering capacity as well as by the amounts of acid and base present, buffering capacity will be considered first.

Buffering constituents of fresh fish muscle

Table I gives the extreme values calculated for β in various pH ranges from the data for fresh haddock's muscle, together with the contributions to the total attributable to its principal constituents in the amounts in which they are known to occur. The individual contributions were obtained by titrating solutions of the particular constituents, knowing the amounts present in 100 g. of fish muscle. Most of the buffering of whole muscle can thus be accounted for, although it is not strictly permissible to add and subtract values of β in this way. The contribution of protein, which, apart from water, is by far the preponderant constituent, amounting to about 16% of the wet muscle, extends over the whole pH range but is always less than half the total buffering power. The buffering curve of Edsall haddock 'myosin', which comprises 70 to 75% of the total protein,[29, 30] exhibited a peak at between pH 6·5 and 7·0, which coincides with that of inorganic phosphate ($pK'_2 = 6·7$). Extraction with bicarbonate

($pK'_1 = 6{\cdot}5$) at pH 7·2 gave similar results,[31] but extraction with 7% lithium chloride, followed by four to six washes with water, removed the peak. The remainder of the protein, including the ' myogen ' fraction and the insoluble stroma, which amounts to about 5% of the total,[32, 33] was assumed to buffer similarly.

Because of the uncertainty of the end-point in the titration of aqueous extracts with indicators containing methyl red, which changes colour between pH 4·2 and 6·3, it had been suspected that the water-soluble constituents of fish muscle included powerful buffers. Liquor pressed from fresh haddock possessed considerable buffering capacity, which was mostly retained when soluble protein was removed by ultra-filtration through collodion sacs or by precipitation with trichloroacetic acid.

The chief water-soluble constituents that could contribute appreciably to the buffering capacity of fresh fish muscle are lactic acid, phosphates and nitrogenous substances, particularly trimethylamine oxide.

Table I

Average buffering powers of the major constituents of fresh haddock muscle (mg.-equiv. of base per 100 g. of muscle per unit pH change)

Component	Concn. (g. per 100 g. fresh muscle)	Range of pH 4·3–4·65	4·65–5·3	5·3–6·0	4·3–6·0	6·0–7·0	7·0–8·0	8·0–9·0	9·0–10·0
' Myosin '	11·6	1·8	0·7	0·9	1·5	0·7	0·7	0·7	1·0
' Myogen ' etc.	4·4	(0·7)	(0·3)	(0·4)	(0·5)	(0·3)	(0·3)	(0·3)	(0·4)
Lactic acid	0·2–0·6	1·1–3·2	0·5–1·4	0·2–0·5	0·5–1·5	0·0	0·0	0·0	0·0
Orthophosphate	0·15	0·0	0·0	0·7	0·4	2·6	1·7	0·5	0·0
Trimethylamine oxide	0·1–0·3	1·0–2·9	0·5–1·5	0·2–0·5	0·5–1·5	0·0	0·0	0·0	0·0
Total	—	4·6–8·6	2·0–3·9	2·4–3·0	3·4–5·4	3·6	2·7	1·5	1·4
Whole fresh muscle	—	5·7–9·5	3·6–5·4	2·9–3·3	3·7–5·6	3·7–4·6	4·0	3·3	3·3

An increase in buffering capacity immediately *post mortem*, attributed by Stansby & Lemon[14] to the breakdown of glycogen to lactic acid ($pK' = 3{\cdot}9$) in unexercised fish,[34] has been confirmed, although the results were irregular. Thus in one case the value of β between pH 4·3 and 4·65 ($\beta_{4{\cdot}3\text{-}4{\cdot}65}$) rose from 4·5 at 30 minutes after death to 5·9 after 28 hours. Fish brought on deck struggling, as is usual commercially, have already exhausted their glycogen and are known to contain 0·2 to 0·6% of lactic acid. There was considerable variation in initial buffering capacity even outside the pH range affected by lactic acid. Thus $\beta_{7{\cdot}0\text{-}10{\cdot}0}$ varied from 3·7 to 5·5 in haddock in *rigor mortis*.

Small quantities of organic phosphates occur in living muscle, e.g. hexose monophosphate ($pK' = 6{\cdot}1$), 0·03%,[35] and phosphocreatine ($pK' = 4{\cdot}5$), 0·5%.[35, 36] These break up within a few hours of death and then buffer as inorganic orthophosphate ($pK_2' = 6{\cdot}7$), 0·15%. Creatine ($pK_b' = 10{\cdot}7$) buffers most strongly at pH 3·3 and therefore exerts only a weak effect above pH 4·3. Fresh North Sea haddock contains from 0·1 to 0·3% of trimethylamine oxide ($pK'_b = 9{\cdot}4$),[3,37] whereas in Arctic cod it varies between 0·1 and 1·0%. These differences are sufficient to account for considerable variation in initial buffering capacity. Marine elasmobranchs such as dogfish and skate usually contain more trimethylamine oxide than marine teleosts, whereas fresh-water teleosts contain very little, and the buffering capacities were of the same order.

Of other nitrogenous extractives which have been reported as constituents of fish (reviewed by Reay *et al.*[32, 33] and Shewan[3]), carnosine ($pK_2' = 6{\cdot}9$), which is important in the physiological pH range for mammals,[27] appears from recent chromatographic work[38] to be absent from cod, which contains, however, 0·15 to 0·30% of anserine ($pK_2' = 7{\cdot}1$). This would account for several per cent. of the value of $\beta_{4{\cdot}3\text{-}6{\cdot}0}$.[23, 27] Neither homologue occurs in fresh-water fish, which contain, however, about 0·2% of histidine ($pK_2' = 6{\cdot}2$).

Shewan, Fletcher, Partridge & Brimley[39] have recently isolated 0·07% of anserine from fresh haddock muscle, and in addition have detected the presence of at least 17 free amino-acids, as well as the expected bases.

Glycine betaine ($pK' = 6{\cdot}2$), of which 0·1% has been reported in cod and about 0·25% in elasmobranchs,[40] was found to have little effect on buffering capacity in these quantities. γ-Butyrobetaine, methylguanidine and various purines etc., which have also been reported as occurring in fish tissue in small quantities, would not be expected to contribute appreciably.

Bicarbonate ($pK_1' = 6{\cdot}5$) contributes little to buffering capacity in mammalian muscle, and above a pH of 7·0 even this effect rapidly decreases.[27]

$\beta_{4{\cdot}3\text{-}6{\cdot}0}$ for captive fresh-water eels was unaffected by an environment of fresh water or sea-water or by a diet of herring or of worms (which contain practically no trimethylamine oxide); it was little more than half that for conger eel. The buffering power for rat's flesh was also independent of fish in the diet. Fulmar petrel, however, had a higher buffering capacity than either rat or rabbit. Although no chemical analyses were made, it was inferred that trimethylamine oxide ingested in the food was not retained by the tissues.

Buffering constituents of fish muscle during spoilage

Fig. 1 shows changes in β in various pH ranges on storage in ice for haddock, which was examined most exhaustively. $\beta_{4{\cdot}3\text{-}4{\cdot}65}$ was greatest and changed most during storage. Although the results were very variable, $\beta_{4{\cdot}3\text{-}4{\cdot}65}$ and $\beta_{4{\cdot}65\text{-}5{\cdot}3}$ decreased between the 8th and 11th day, coinciding roughly with the disappearance of trimethylamine oxide; $\beta_{9{\cdot}0\text{-}10{\cdot}0}$ increased, however, corresponding with the production of trimethylamine ($pK_b' = 4{\cdot}1$),[18, 19] which would account for a value of about 2·5 after about the 16th day in ice. Ammonia itself ($pK_b' = 4{\cdot}7$), which is produced in stale fish, also buffers in this range. So do most amino-acids that might be expected to be released in advanced putrefaction. Between pH 5·3 and 9·0, buffering was weaker and less variable, decreasing slowly during storage.

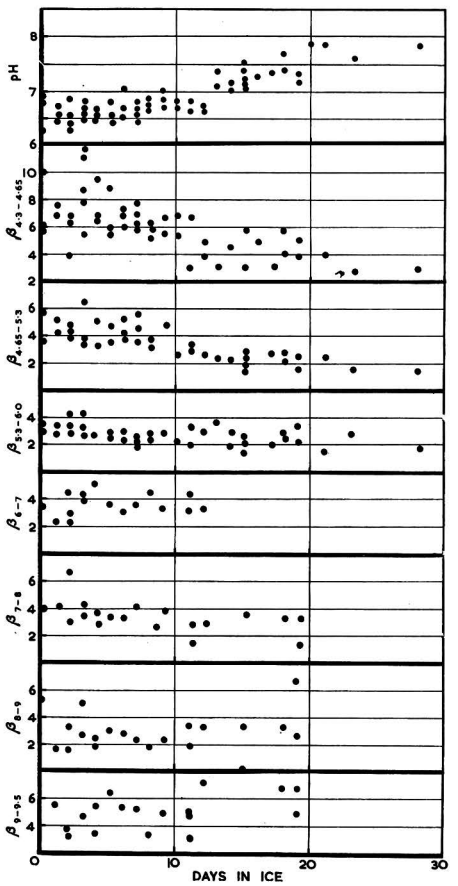

FIG. 1.—*Effect of stowage in ice on pH and buffering capacity of muscle suspensions of haddock* (Gadus aeglefinus)

Sharp[41] found that lactic acid did not disappear from haddock's muscle in 12 days at 0°. Collins,[42] however, postulated a reaction in the tissue between lactic acid and trimethylamine oxide, with the formation of acetic acid ($pK' = 4{\cdot}3$), which would contribute to $\beta_{4{\cdot}3\text{-}6{\cdot}0}$, trimethylamine and carbon dioxide.

Other marine teleosts behaved similarly. Fig. 2 gives the values for β in different ranges for whiting, and $\beta_{4{\cdot}3\text{-}4{\cdot}65}$ for a number of other species. $\beta_{4{\cdot}3\text{-}6{\cdot}0}$ is plotted in Fig. 3 because of its relevance to the 'freshness index' of Stansby & Lemon.[14] Dogfish and skate had a high initial value which fell to a quite low level, although there was considerable variation from one experiment to another. $\beta_{4{\cdot}3\text{-}4{\cdot}65}$ for fresh-water fish and warm-blooded animals was rather low and there was no appreciable alteration during storage. This behaviour would be expected if the decrease in buffering were largely due to the disappearance of trimethylamine oxide. Mammals, however, contain more lactic acid than fish, and carnosine in addition.

In an attempt to separate the effects of bacterial decomposition and autolysis, pieces of cod muscle were kept in stoppered tubes under aseptic conditions. They developed no putrefactive odours even after 10 weeks at 0°, and the buffering capacity and pH showed no marked trend compared with control pieces and iced fish (see Fig. 4). Aberrant values were invariably associated with contamination of the sample, although the degree of infection varied. Such samples often had a high buffering capacity, perhaps because the particular bacteria responsible were not reducers of trimethylamine oxide.

The curve of A value with time shown in Fig. 3 is of a form very roughly parallel with

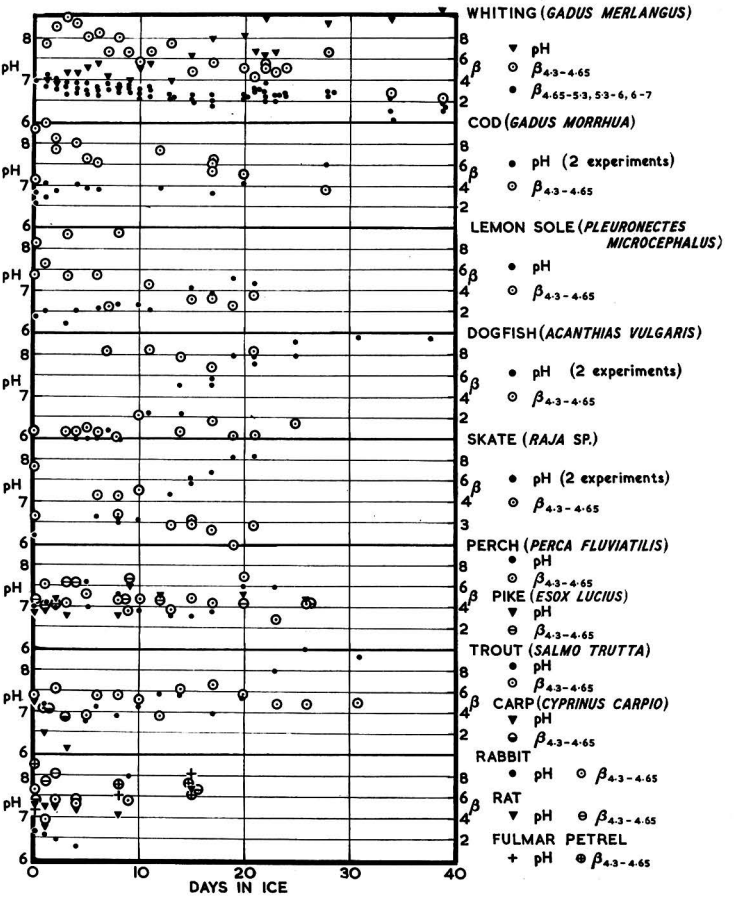

FIG. 2.—*Effect of stowage in ice on pH and buffering capacity of muscle suspensions of various fish and other animals*

that normally obtained for trimethylamine oxide content; but the variation from fish to fish and from one experiment to another is so great that values for fresh fish were sometimes as low as those for fish 14 days in ice and on the verge of putrefaction. No combination with *B* value could convert the measurement of buffering capacity into a reliable criterion of freshness. The results of Stander[43] for a species of hake led to similar conclusions.

Despite initial hopes, the test could assist in the estimation neither of deterioration in frozen fish nor of proteolysis of hake associated with protozoal infection described by Fletcher, Hodgkiss & Shewan.[44] However, certain dried fish-albumen preparations, of which the value of $\beta_{4\cdot3-6\cdot0}$ was 8·0, were presumed, rightly, to have been partly hydrolysed. The value for fresh fish viscera was about 20.

Post-mortem changes in the pH of fish muscle

Fish contains considerably less glycogen and has a higher buffering capacity than mammalian muscle, which normally reaches a pH of 5·8. However, the pH of halibut may fall as low[23] as 5·5.

Typical results for the pH changes of haddocks and whiting immediately *post mortem*, obtained by the use of a probe glass electrode, are shown in Fig. 5. A dogfish 1½ hours after catching and gutting had a pH of 6·4. Hake, which Benson[5] reported to be slightly alkaline,

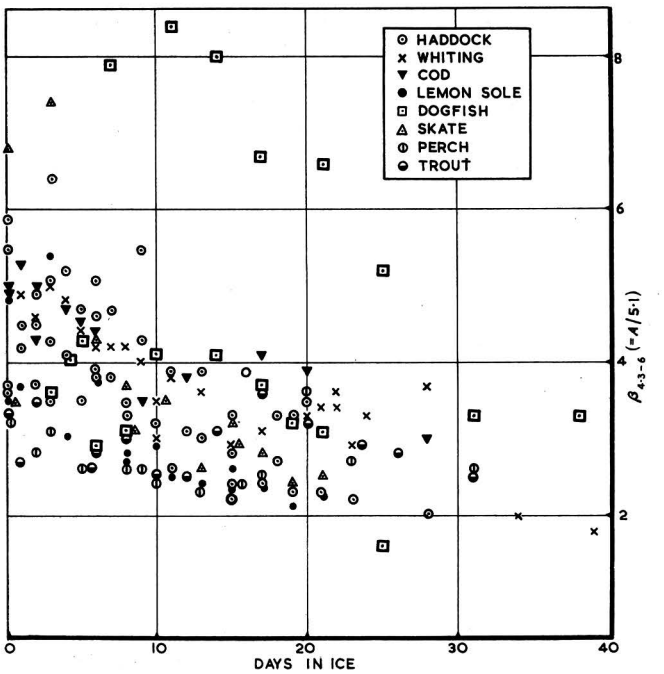

FIG. 3 (*above*).—*Titration values between pH 6·0 and 4·3 for various species of fish*

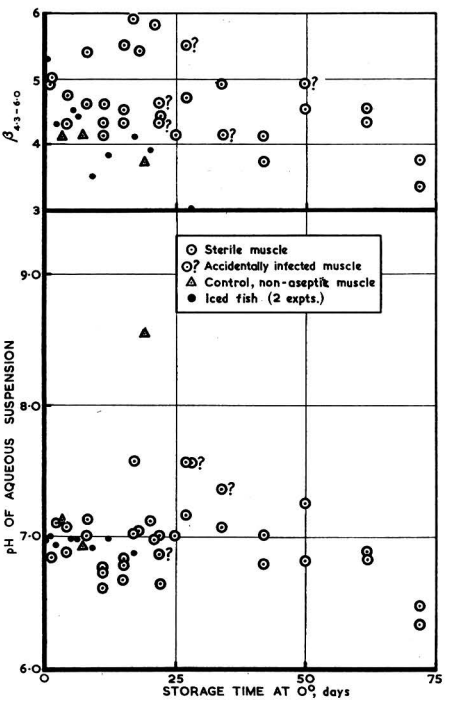

FIG. 4 (*left*).—*Buffering capacity and pH of sterile cod's muscle*

was found some hours after catching to be at pH 6·8. A Norway lobster (*Nephrops norvegicus*) was at pH 6·5 at death and was unaltered after an hour.

Fig. 1 shows that there was a considerable variation in the pH of a suspension of fresh fish muscle. The values were usually only slightly higher than those of the original fish muscle. Hjorth-Hansen[23] found dilution had an effect of at least 0·1 pH unit.

The approximate ranges of initial pH of various types of fish were as follows: gadoid species, 6·3 to 7·0; flat fish, 6·4 to 6·6; elasmobranchs, 6·2 to 6·5; eels and herrings, 6·1 to 6·4; fresh-water fish, 6·9 to 7·3.

Post-mortem pH changes could be followed by using muscle suspensions. Thus, specimens of haddock, whiting and lemon sole minced 30 minutes after death still had pH values of 7·02, 7·06 and 6·8 respectively. Another lemon sole from the same batch fell in pH to 5·9

after 6 hours, whereas that of an exercised fish, which had presumably depleted its glycogen reserves, reached only 6·86. Carp had a pH of 7·3 before, 6·4 during, and 6·5 after *rigor*.

When fish were stored in ice after capture, there was usually little or no further change in pH (after full *rigor* was attained) for about 10 days. By this time the fish were fairly stale. After this, the production of ammonia and amines caused the pH to rise to between 7·5 and 8·0 after 20 to 25 days, when the fish were quite putrid and inedible. Dogfish, in which the breakdown of urea releases additional ammonia,[45] reached pH 8·5. These increases can be completely accounted for on the basis of the buffering capacity and the quantities of base produced.[45, 46]

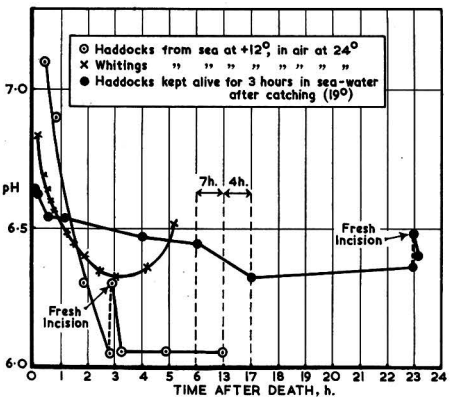

FIG. 5.—*Changes of pH in trawled fish immediately post mortem*

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References

[1] Reay, G. A. & Shewan, J. M., *Advanc. Food Res.*, 1949, **2**, 343
[2] Shewan, J. M., *J. R. sanit. Inst.*, 1949, **69**, 394
[3] Shewan, J. M., *Symp. biochem. Soc.*, No. 6, 1951, 28
[4] Furusawa, K. & Kerridge, P. M. T., *J. Mar. biol. Ass. U.K.*, 1927, **14**, 657
[5] Benson, C. C., *J. biol. Chem.*, 1928, **78**, 583
[6] Schlie, K., *Kälteindustrie*, 1934, **31**, 115
[7] Schonberg, F., *Vorratspfl. u. Lebensmittelforsch.*, 1938, **1**, 133
[8] Cutting, C. L., *Rep. Fd Invest. Bd, Lond.*, 1939, p. 89
[9] Boury, M. & Schwinte, J., *Rev. Trav. Off. Pêches marit.*, 1935, **8**, 282
[10] Dyer, W. J., Sigurdsson, G. S. & Wood, A. J., *Food Res.*, 1944, **9**, 183
[11] Elliott, R. P., *Food Res.*, 1947, **12**, 87
[12] Kondrup, M., *Ministry of Fish Res. Lab., Copenhagen*, 1948, Rep. No. 73
[13] Notevarp, O. & Hjorth-Hansen, S., *Aarsberetn. Norg. Fish.*, 1932, **3**, 39
[14] Stansby, M. E. & Lemon, J. M., *Industr. Engng Chem. (Anal.)*, 1933, **5**, 208
[15] Griffiths, F. P. & Stansby, M. E., *Trans. Amer. Fish. Soc.*, 1934, **64**, 401
[16] Fitzgerald, G. A. & Conway, W. S., *Amer. J. publ. Hlth*, 1937, **27**, 1094
[17] Stansby, M. E. & Fitzgerald, G. A., *Industr. Engng Chem. (Anal.)*, 1939, **31**, 290
[18] Cutting, C. L., *Rep. Fd Invest. Bd, Lond.*, 1937, p. 78
[19] Cutting, C. L., *Rep. Fd Invest. Bd, Lond.*, 1938, p. 89
[20] Beatty, S. A. & Gibbons, N. E., *J. biol. Bd Can.*, 1937, **3**, 77
[21] Nickerson, J. T. & Proctor, B. E., *J. Bact.*, 1935, **30**, 383
[22] Collins, V. K., Kuchel, C. C. & Beatty, S. A., *J. Fish. Res. Bd Can.*, 1941, **5**, 203
[23] Hjorth-Hansen, S., *Rep. Norwg. Fisheries Res. Lab.*, 1943, **1**, No. 4
[24] Hacker, R., *Z. Lebensmitt Untersuch.*, 1950, **90**, 361
[25] Reay, G. A. & Shewan, J. M., 'The care of the trawler's fish', 1949, Dep. sci. industr. Res., Lond., Food Investig. Leafl. No. 3 (London: H.M.S.O.)
[26] Edsall, J. T., *J. biol. Chem.*, 1930, **89**, 289
[27] Bate-Smith, E. C., *J. Physiol.*, 1938, **92**, 336
[28] van Slyke, D. D., *J. biol. Chem.*, 1922, **52**, 525
[29] Reay, G. A. & Küchel, C. C., *Rep. Fd Invest. Bd, Lond.*, 1937, p. 93
[30] Dyer, W. J., French, H. V. & Snow, J. M., *J. Fish. Res. Bd Can.*, 1950, **7**, 585
[31] Meyer, K., *Biochem. Z.*, 1933, **266**, 137
[32] Reay, G. A., Cutting, C. L. & Shewan, J. M., *J. Soc. chem. Ind., Lond.*, 1943, **62**, 77
[33] Reay, G. A., Cutting, C. L. & Shewan, J. M., 'The Nation's Food', Ed. A. L. Bacharach & T. Rendle, 1946, Part VI, p. 269 (London: Society of Chemical Industry)
[34] Leim, A. H., McLeod, J. J. R. & Simpson, W. W., *Contr. Canad. Biol.*, 1927, **3**, 1459
[35] Tarr, H. L. A., *J. Fish. Res. Bd Can.*, 1950, **7**, 608
[36] Bate-Smith, E. C., private communication
[37] Reay, G. A., *Rep. Fd Invest. Bd, Lond.*, 1938, p. 87
[38] Yudaev, H. A., *Trans. Acad. Sci. U.S.S.R.*, 1950, **70**, 279
[39] Shewan, J. M., Fletcher, L I., Partridge, S. M. & Brimley, R. C., *J. Sci. Fd Agric.*, 1952, **3**, 394
[40] Kapeller-Adler, R. & Kraal, J., *Biochem. Z.*, 1930, **221**, 437; **224**, 364
[41] Sharp, J. G., *Proc. roy. Soc.*, 1934, [B] **114**, 506
[42] Collins, V. K., *J. Fish. Res. Bd Can.*, 1941, **5**, 197
[43] Stander, G. H., *4th Ann. Rep. Dir. Fish. Ind. Res. Inst., Cape Town*, 1951, p. 5
[44] Fletcher, L. I., Hodgkiss, W. & Shewan, J. M., *Fish. News, Aberd.*, 1951, No. 2007, 10
[45] Shewan, J. M., *Rep. Fd Invest. Bd, Lond.*, 1938, p. 79
[46] Shewan, J. M., *Rep. Fd Invest. Bd, Lond.*, 1937, p. 75

AMINO-ACIDS IN NEW ZEALAND CHEDDAR CHEESE: THEIR POSSIBLE CONTRIBUTION TO FLAVOUR

By JACK C. DACRE

1. Paper partition chromatographic analysis of four maturing New Zealand Cheddar cheeses showed them to contain the following ninhydrin-reacting compounds: glutamic acid, lysine and/or arginine, leucine and *iso*leucine, valine, phenylalanine, alanine, aspartic acid, glycine, glutamine, asparagine, threonine, methionine, a peptide, and sometimes tyrosine, serine and proline, in the water-soluble nitrogenous fraction.

2. All the amino-acids increased steadily in amount as the cheeses matured, except for tyrosine, the concentration of which fluctuated in the different cheeses, probably as a result of the decarboxylating activity of some of the lactobacilli present in the cheeses.

3. Comparison of the times of appearance of the individual amino-acids with flavour development indicated no correlation between a particular acid or acids and the development of Cheddar cheese flavour.

4. The individual amino-acids and their corresponding amines, as well as mixtures of the amino-acids as found in mature cheese, did not possess any aroma or taste which might suggest that they contributed to the typical flavour of Cheddar cheese.

Introduction

Many workers during the past fifty years have investigated the process of Cheddar cheese ripening; this results in the gradual change in the cheese curd from toughness to plasticity and in the development of the aroma and taste that together constitute typical cheese flavour. The results of the earlier work showed than the main change occurring during the ripening process was a gradual degradation of the casein under the influence of the rennet enzymes and bacterial enzymes, and possibly the enzymes present in the original milk. These processes resulted in the formation of a complex mixture of peptones, polypeptides and amino-acids, with an accompanying change in the physical nature of the cheese curd, which gradually became more plastic. In recent years, a more precise definition of some of the casein-degradation products has been made by the use of microbiological-assay and paper partition chromatography techniques. Kosikowsky[1] reviewed the work done on Cheddar cheese up to 1950.

Although much has been learned about the gross chemical changes involved in cheese ripening, no one has so far succeeded in defining the substance or substances that confer flavour on the cheese. Several workers (e.g. Sherwood[2]) have obtained indications that certain strains of lactic acid bacteria, when added to the cheese milk, influence the type of flavour developed as well as its rate of development. But this purely empirical result has left the question of the identity of Cheddar cheese flavour at the stage at which the problem of butter flavour rested before the discovery that diacetyl (butane-2 : 3-dione) was the flavouring substance produced in butter-starter cultures.

In types of cheese other than Cheddar, there have been suggestions that amino-acids contribute to the characteristic flavour. Virtanen & Kreula[3] and Virtanen, Kreula & Nurmikko[4] reported that proline was responsible for the characteristic sweetish taste of Emmentaler cheese. They also considered it significant that amino-acids with a bitter taste, such as arginine and methionine, decreased in concentration as the ripening progressed. Storgårds & Hietaranta,[5] on the other hand, attributed the flavour of Emmentaler cheese to its high content of the 'sour-tasting' aspartic and glutamic acids. Demeter[6] found that the taste of this type of cheese was due to proline and the odour due to the decomposition products of leucine. The conclusions of these various workers are not in agreement and it is evident that further investigation is necessary to define the relationship of the amino-acids to Emmentaler cheese flavour.

In recent years the possible connexion of amino-acids with Cheddar cheese flavour has attracted the attention of many workers, and newer methods of amino-acid analysis have made possible the more detailed investigation of the liberation of these compounds during cheese ripening. Harper & Swanson[7] measured, by microbiological assay, the concentrations of nine amino-acids in American Cheddar cheese of ages varying from two weeks to four years. They found that the concentration of glutamic acid, glycine, leucine, *iso*leucine, lysine and valine increased with the age of the cheese. They also found that the addition of mixtures of these amino-acids to fresh curd gave a taste (but not an aroma) reminiscent of Cheddar cheese. Baker & Nelson[8] studied flavour development in American Cheddar cheese made from batches of curd to each of which one of 19 different amino-acids had been added. They found no consistent improvement in flavour that could be attributed to the presence of any of the acids, except with serine, where there was a slightly improved flavour. Since, however, the presence

of serine led to an increased bacterial growth in the cheese, the direct influence of the amino-acid on flavour was doubtful. Histidine gave an undesirable flavour.

Several workers, using the paper partition chromatographic technique,[1, 9, 10] determined the concentrations of amino-acids and amines (i.e. the ninhydrin-reacting compounds) occurring in American Cheddar chéeses made from both raw and pasteurized milks. Other workers[11–13] determined the time and order of appearance and rate of production of the amino-acids in American Cheddar cheese in the hope of being able eventually to correlate the information so obtained with the ripening of the cheese and the development of the flavour. Simonart & Mayaudon[14] reported results of an extensive chromatographic investigation of the amino-acids present in 12 different types of cheeses (not including Cheddar) as well as the times of appearance of the acids in Herve cheese. Because the amino-acids histidine, citrulline and asparagine were found in hard and not in soft curd cheeses, and α-aminobutyric acid, β-alanine and peptides were present in soft and not in the hard types of cheese, these workers concluded that these compounds played a significant part in the two different types of cheese.

The present work was undertaken with the intention of reviewing the possible influence of the amino-acids and their derivatives on the flavour of New Zealand Cheddar cheese. The elegant paper partition chromatographic technique of Consden, Gordon & Martin[15] was used to determine the time and order of appearance of amino-acids in several typical Cheddar cheeses of the New Zealand type. The taste and aroma of the individual amino-acids and amines, and of mixtures of acids corresponding to those found to occur in the cheese, were investigated in the hope of linking taste or aroma of cheese with that of any of the compounds found to be present by analysis.

Materials and methods

Cheeses

The four cheeses analysed were 10-lb. loaves made from flash-pasteurized milk and taken directly from the press the day after manufacture. Two of the cheeses were made in the experimental factory of the Dairy Research Institute and two were made in commercial cheese factories. On removal from the press, the cheeses were immediately cut up into pieces approximately 350 g. in weight. The pieces were waxed and stored in glass jars with loose-fitting lids in the laboratory at room temperature.

Preparation of samples for analysis

A 10-g. sample cut from the interior of one of the waxed pieces of cheese was ground in a mortar with 50 ml. of distilled water. Of this mixture 20 ml. was centrifuged to separate the fat and then a small volume (about 2 ml.) of the aqueous layer was carefully pipetted off and several drops immediately applied as a small spot on a 22-in. × 18-in. sheet of filter paper (Whatman No. 1) for two-dimensional paper partition chromatographic analysis.

Paper partition chromatographic analysis

The technique followed was essentially that of Consden, Gordon & Martin.[15] The upper phase of a mixture of *n*-butanol (25 parts), distilled water (25 parts) and glacial acetic acid (6 parts) was used for the first run. For the second run, phenol saturated with water was used, with the addition of 0·1% of concentrated ammonia solution to the trays in the bottom of the cabinet. The chromatograms were dried at room temperature after each run, then evenly sprayed with a 0·1% ninhydrin solution in *n*-butanol, dried and finally developed by heating in an 80° oven for five minutes. The intensity of the resulting ninhydrin colour reaction was used to obtain a rough quantitative estimation of the amounts of the individual amino-acids present. In all cases identity of the compounds was confirmed by the preparation of duplicate chromatograms, either with the addition of the known compound or compounds to the test solution and checking for an increase in individual colour intensity of the ninhydrin reaction, or running chromatograms of the known compounds and comparing the individual R_F values and characteristic ninhydrin colours. With glutamine and asparagine, acid hydrolysis of the test solution gave further confirmation of their identity.

Amino-acids and amines examined

The amino-acids (in their L-, D- or DL-forms) as found to occur in Cheddar cheese (see Table I), together with their corresponding amines, were examined by a panel, both for taste and volatility or aroma, in their pure crystalline or liquid forms, as a 1% aqueous solution

and in a 1·5% sodium chloride solution. The following amines were not examined: β-aminopropionamide (from asparagine), aminoisopropyl alcohol (from threonine) and γ-methylmercaptopropylamine (from methionine). In addition, the following amino-acids and related compounds were included in the tests: DL-α-amino-*n*-butyric acid, L- and DL-citrulline, cysteine, L-cystine, 3 : 4-dihydroxyphenylalanine, L-histidine, histamine, DL-norleucine, L- and DL-ornithine, L-hydroxyproline, putrescine hydrochloride, taurine, L- and DL-tryptophan, and DL-norvaline.

Results

The results of the chromatographic analyses of the four cheeses for amino-acids are summarized in Tables I and II.

Table I

Relative concentrations of the various amino-acids in New Zealand Cheddar cheese as deduced by visual appraisal of the colours produced with ninhydrin on two-dimensional chromatograms

Age of cheese	7 days				14 days				1 month			
Cheese number	1	2	3	4	1	2	3	4	1	2	3	4
Amino-acids												
Glutamic acid	2+	2+	2+	2+	2+	2+	2+	2+	2+	3+	2+	2+
Lysine and arginine	±	±	±	+	+	±	±	+	+	+	+	2+
Leucine and *iso*leucine	+	+	2+	+	+	2+	2+	2+	2+	2+	2+	2+
Tyrosine	±	+	±		±	±	±	±	+	±	−	+
Valine	±	+	±	±	±	2+	+	+	+	2+	+	2+
Phenylalanine		±	+		±	+	+	+	+	2+	+	2+
Alanine		±	±	±	+	±	+	±	+	2+	+	+
Aspartic acid		±				±	±	±	±	+	±	+
Glycine		±	±			±	±	±		+	+	+
Glutamine								±	±	±	±	2+
Asparagine											±	+
Methionine							±			±	+	
Threonine												±
Serine												±
Proline												±

Age of cheese	2 months				3 months				6 months			
Cheese number	1	2	3	4	1	2	3	4	1	2	3	4
Amino-acids												
Glutamic acid	2+	3+	3+	3+	3+	3+	3+	3+	4+	4+	4+	4+
Lysine and arginine	+	+	+	2+	2+	2+	2+	2+	3+	3+	3+	3+
Leucine and *iso*leucine	2+	3+	3+	3+	2+	4+	4+	3+	4+	4+	4+	4+
Tyrosine	+	−	−	+	+	±	−	+	±	−	−	2+
Valine	2+	2+	2+	3+	2+	2+	3+	3+	4+	4+	4+	3+
Phenylalanine	+	2+	2+	2+	2+	2+	2+	2+	3+	3+	3+	2+
Alanine	+	2+	2+	+	+	2+	2+	2+	3+	3+	3+	3+
Aspartic acid	±	+	+	+	+	+	+	+	2+	+	2+	2+
Glycine	±	+	2+	+	+	2+	2+	2+	2+	3+	3+	2+
Glutamine	±	+	2+	2+	+	2+	2+	2+	3+	3+	3+	2+
Asparagine	±		±	+	±	+	+	+	2+	2+	2+	2+
Methionine	±	+	2+		±	+	2+	±	+	2+	3+	+
Threonine				±			+	+	+	±	2+	2+
Serine				±				+		+		+
Proline				±				±	±			+
Peptide		±	±	±		±	±	±	±	±	+	±

Concentrations present are indicated as follows:

− = not found
± = up to about 0·5 mg./g.
\+ = about 0·5–1 mg./g.
2+ = about 1–2·5 mg./g.
3+ = about 2·5–5 mg./g.
4+ = greater than about 5 mg./g.

It is evident from Table I that although the general pattern of amino-acid liberation in the four cheeses analysed was similar, there were considerable variations in detail from cheese to cheese, especially in the times of appearance of any particular amino-acid (see Table II). No correlation was observed between the appearance of any particular acid and the development of the typical Cheddar cheese flavour, which became evident as usual in 14 to 21 days

and then gradually increased in intensity. All the amino-acids set out in Table I, with the exception of tyrosine, increased steadily in concentration as ripening progressed; tyrosine, as has previously been shown (see Dacre[16]), is decarboxylated by *Lactobacillus brevis* strains normally present in New Zealand Cheddar cheese.

The chromatographic analysis also showed that amines (apart from tyramine) were absent from the cheeses; this result was to be expected, since the lactic acid bacteria that predominate in the cheese flora do not, in general, possess amino-acid-decarboxylating enzyme systems. Soluble ninhydrin-reacting peptides did not appear until the cheeses were two months old, when there was a trace of a compound corresponding in R_F values with the 'nephrosis peptide' described by Dent.[17] No evidence was found for the presence of histidine and citrulline in the four cheeses examined. This is in agreement with the detailed examinations reported by Kosikowsky[11] for American Cheddar cheese, but not in agreement with the conclusions of Simonart & Mayaudon[14] relating to the qualitative differences in amino-acid composition between hard and soft curd types of cheese.

Table II

Times of appearance (in days) of the individual amino-acids in New Zealand Cheddar cheese

Cheese number ..	1	2	3	4
Amino-acids				
Glutamic acid	1	1	1	1
Lysine and arginine	1	7	6	1
Leucine and *iso*leucine	1	1	3	4
Tyrosine*	7	1	6	13
Valine	7	7	6	6
Phenylalanine	10	7	6	13
Alanine	10	3	3	6
Aspartic acid	17	7	14	13
Glycine	35	7	6	13
Glutamine	50	41	18	13
Asparagine	71	80	29	20
Methionine	57	28	14	103
Threonine	124	101	76	25
Serine		171		27
Proline	117			40
Peptide	131	59	62	33

* Variable results: see Table I

Although cystine and tryptophan were not identified on any of the chromatograms, their presence in very slight amounts was demonstrated by the nitroprusside test for –SH groups[18] and by the *p*-dimethylaminobenzaldehyde reaction.[19] Since flavour substances are sometimes effective in very small traces, this finding emphasizes the danger of eliminating from consideration any substance merely because it may not be detected by any one method of chemical or microbiological analysis.

Examination of the pure amino-acids and their amines

As a further test of the possible contribution of the amino-acids to cheese flavour, all the acids found by chromatographic analysis to be present in the cheeses, most of the corresponding amines and many related compounds were examined organoleptically both for taste and aroma. None of the compounds had either the taste or aroma of Cheddar cheese; they were all either tasteless, very slightly sweet or bitter. These results are in agreement with those of Crocker,[20] who found that the amino-acids, their sodium salts and hydrochlorides were without odour, and of Murray & Baker,[21] who concluded that no one amino-acid contributed significantly to the taste of enzymic protein hydrolysates.

It was evident from the present experiments that no one amino-acid, or its sodium salt or amine, had any direct connexion with Cheddar cheese flavour.

In a further trial, mixtures of the amino-acids present in cheese, as indicated by the chromatographic analyses, were added to fresh cheese curd. An amino-acid mixture corresponding to that found in a six-months-old cheese gave no flavour to fresh curd. Even after being kept for a month under cheese-curing conditions, this experimental curd acquired no more cheese flavour than a control curd to which no amino-acids had been added.

Discussion

The results obtained in this work do not support the suggestions that amino-acids or amines, either singly or in admixture, have a direct connexion with the taste or aroma (i.e. the flavour) of Cheddar cheese. It is understandable that protein degradation, formation of volatile acids and lactic acid, and production of tyramine should all correlate generally with the gradual appearance of that basic flavour which is typical of all hard-pressed acid cheeses such as Cheddar and Cheshire. But this does not mean that any of the substances so far investigated are themselves responsible for the flavour. Apart from the findings of Harper & Swanson,[7] which have not yet been confirmed, no clue has so far been discovered that points towards a solution of this complex problem.

The fact that certain bacteria, notably strains of lactobacilli, when present in the cheese cause an earlier and more intense development of the ' cheesy ' taste and odour suggests that bacterial enzymes are responsible for the formation of flavour substances. Hence the two most promising lines of attack would appear to be: (i) an attempt to isolate from mature Cheddar cheese the typical flavouring substance or substances and to define them chemically (see Suzuki, Hastings & Hart[22]); (ii) an investigation of the metabolism of the flavour-producing lactobacilli in the hope of obtaining a clue to the possible nature of the flavouring substances.

Difficulties from the chemical point of view are enhanced by the probability that the flavouring substances are present to the extent of only a few parts per million of cheese. Progress may come either empirically, with the discovery of some method of controlling cheese flavour without a knowledge of the chemical substances concerned, or, more precisely, with a discovery of the chemical nature of the substance or substances responsible for the typical taste and aroma of mature Cheddar cheese.

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References

[1] Kosikowsky, F. V., *J. Dairy Sci.*, 1951, **34**, 228

[2] Sherwood, I. R., *J. Dairy Res.*, 1937, **8**, 224

[3] Virtanen, A. I. & Kreula, M. S., *Meijerit. Aikakausk.*, 1948, **10**, 13

[4] Virtanen, A. I., Kreula, M. S. & Nurmikko, V. T., *Proc. int. Dairy Congr.* (No. 12, Stockholm), 1949, **2**, 268

[5] Storgårds, T. & Hietaranta, M., *Proc. int. Dairy Congr.* (No. 12, Stockholm), 1949, **2**, 227

[6] Demeter, K. J., *Süddtsch. Molkereiztg*, 1949, **70**, 862; *Chem. Abstr.*, 1952, **46**, 9226e

[7] Harper, W. J. & Swanson, A. M., *Proc. int. Dairy Congr.* (No. 12, Stockholm), 1949, **2**, 147

[8] Baker, R. J. & Nelson, F. E., *J. Dairy Sci.*, 1949, **32**, 769

[9] Block, R. J., *J. Dairy, Sci.*, 1951, **34**, 1

[10] Trautman, J. C., Hansen, H. C. & Hartwell, W. V., *Proc. Amer. Dairy Sci. Ass.* (32nd Ann. Meeting, Western Div., Montana), 1951, p. 74

[11] Kosikowsky, F. V., *J. Dairy Sci.*, 1951, **34**, 235

[12] Honer, C. J. & Tuckey, S. L., *J. Dairy Sci.*, 1951, **34**, 475

[13] Fagen, H. J. & Hussong, R. V., *J. Dairy Sci.*, 1952, **35**, 479

[14] Simonart, P. & Mayaudon, J., *Neth. Milk Dairy J.*, 1952, **6**, 1

[15] Consden, R., Gordon, A. H. & Martin, A. J. P., *Biochem. J.*, 1944, **38**, 224

[16] Dacre, J. C., *J. Dairy Res.*, 1953, **20**, 217

[17] Dent, C. E., *Biochem. J.*, 1948, **43**, 169

[18] Barnicoat, C. R., *J. Dairy Res.*, 1950, **17**, 209

[19] Erekson, A. B., *J. Dairy Sci.*, 1949, **32**, 704

[20] Crocker, E. C., *Proc. Symp. Monosodium Glutamate* (No. 1, Chicago), 1949, p. 25

[21] Murray, T. K. & Baker, B. E., *J. Sci. Fd Agric.*, 1952, **3**, 470

[22] Suzuki, S. K., Hastings, E. G. & Hart, E. B., *Res. Bull. Wis. agric. Exp. Sta.*, No. 11, 1910

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